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Are There any Centrally Controlled Sympathetic Inhibitory Fibres to the Musculature of the Intestine?

By

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Abstract

CELANDER, O. Are there any centrally controlled sympathetic inhibitory fibres to the musculature of the intestine? *Acta physiol. scand.* 1959. *47*. 299—309. — Intestinal blood flow and motility were recorded during catechol amine injections and low-frequency stimulations of the preganglionic sympathetic outflow to the gut. The inhibitory response and the intestinal vasoconstriction always started simultaneously when catechol amines were administered *via* the blood stream. In contrast to this, the sympathetic stimulation induced an intestinal inhibition that was characterized by an often considerable latent period and a late maximum when compared with the prompt vasoconstrictor response. Prompt inhibition of the intestine was generally seen only at stimulation frequencies above the 'physiological' range, under which circumstances a considerable overflow of the transmitter from vasoconstrictor nerve endings is known to take place. Further, when a reduction of intestinal blood flow, comparable to that induced by low-frequency sympathetic stimulation, was induced by partial occlusion of the supplying artery an almost identical intestinal inhibition was commonly seen. Therefore, the inhibitory response to preganglionic sympathetic stimulation may be due simply to the inevitable reduction of intestinal blood supply and (or) to a local 'overflow' of the vasoconstrictor fibre transmitter.

Since the extensive study by BAYLISS and STARLING (1899) it is almost generally agreed that both divisions of the autonomic nervous system, more or less in a reciprocal way, modify the activities of the intestinal smooth muscle cells. The inhibitor action of the sympathetic is conceived to be effected by specific adrenergic neurons. This type of innervation is also accepted to explain the inhibitor response of the musculature of the uterus and the bronchi.

The physiological evidence in favour of specific sympathetic inhibitory fibres lies mainly in the well-known fact that stimulation of the sympathetic outflow and the administration of the sympathetic neuro-hormones inhibits the intestinal motility. However, sympathetic stimulation includes an activation of the adrenergic vasoconstrictor nerves, and remarkably few experiments have been carried out to study the relations between intestinal blood flow and motility. BAYLISS and STARLING (1899) showed that 'cutting out' the heart did not prevent the inhibition following stimulation of the splanchnics although circulation was then abolished. The same independence of circulatory changes was demonstrated by FINKLEMAN (1930) by *in vitro* experiments and, more recently, in meticulously controlled experiments by GARRY and GILLESPIE (1955). The existence of vasoconstrictor nerve fibres should, however, not be ignored even in these *in vitro* studies, since a release of the adrenergic transmitter at the vasoconstrictor nerve endings might occur in excess of the local inactivation with an 'overflow' as a result. In fact, what FINKLEMAN showed was that following stimulation of the mesenteric nerves at 20—40/sec 'a substance appears in the fluid — — — — which has the power of inhibiting a second piece of gut'.

There is now good evidence that the frequency of impulses normally transmitted by sympathetic neurons seldom if ever exceeds 10/sec with a basal discharge rate of only up to 1—2 impulses per sec. This subject has recently been discussed at length by FOLKOW (1952, 1956) and CELANDER (1954). It is also known that no significant overflow of the transmitter occurs unless abnormally high frequencies of stimulation are used (CELANDER 1954), recently confirmed by BROWN and GILLESPIE (1957). The functional capacity of various tissues to eliminate the sympathetic neurohormones is wide (CELANDER and MELLANDER 1955). On the other hand, once the transmitter has entered the extracellular fluid space it seems to be fairly stable (CELANDER and MELLANDER 1956). The combined evidence therefore indicates that the liberation and destruction of the sympathetic transmitter occur close to the neuroeffector junctions, and that normally there is only a functionally insignificant diffusion to noninnervated neighbouring cells.

The present investigation was undertaken in order to study whether the response of the musculature of the small intestine, following stimulation of the splanchnic nerves, had the characteristics of a true 'neurogenic' effect, or if changes in blood flow and excessive release of the adrenergic transmitter at vasoconstrictor nerve endings could be offered as more plausible explanations.

A preliminary account of this study was published in 1956.

Methods

Operative procedures. Experiments were performed on cats anaesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg). The abdomen was opened in the midline and an intestinal loop was isolated by tying thick ligatures around the gut. The positions of these ligatures were chosen with regard to the distribution of the mesenteric vessels. As a rule, the isolated loop consisted of the caudal part of the duodenum and proximal part of the jejunum, altogether some 15 cm. The rest of the gastro-intestinal tract was removed as well as the great omentum and the spleen. These operative procedures had to be done carefully so that the superior mesenteric artery and the portal vein were not obstructed or interfered with. Great care was taken to leave the hepatic artery and the adjacent nervous tissue undamaged. Thus the intestinal loop had its main blood supply and its innervation intact.

To enable as complete an activation as possible of the preganglionic sympathetic nerve fibres the greater and lesser splanchnic nerves were isolated on both sides, carefully dissected free and cut as high up in the thoracic cavity as was possible. On the right side this preparation was rather difficult owing to the close neighbourhood to the adrenal gland and, as a rule, had to be done with the aid of magnifying glasses. The right splanchnic nerves were brought under the inferior caval vein over to the left side to join the left splanchnic nerves. They could then all be put on one stimulating electrode.

Ligatures were placed around the adrenal glands to enable a 'tying off' at both poles so that the medullary secretion following upon splanchnic nerve stimulation would not interfere with the neurogenic effects on the intestinal loop. The tying of these ligatures, however, was postponed as long as possible in the course of the preparation to avoid any possible detrimental effects of corticoid deficiency.

In order to be able to alter mechanically the blood flow through the isolated loop, an adjustable clamp was placed around the aorta just above the superior mesenteric artery.

Both vagal nerves were cut in the neck. Injections of catechol-amines were made intravenously or into the abdominal aorta through a catheter in the splenic artery.

Blood flow. After heparin had been given a polythene tube was tied into the distal end of the portal vein and the blood flow was diverted to an optical drop-recorder operating an ordinate-writer. The blood was then reinfused into the animal through a jugular vein. In the records the heights of the ordinates are inversely proportional to the rates of blood flow.

Intestinal motility. The ordinary rubber balloon technique for recording intestinal tone and motility was considered unsuitable for several reasons, the main one being its possible interference with the circulation of the mucosal layer. Instead, the loop was filled with Tyrode-solution and connected to a volume recorder. This system was so arranged that the pressure was kept approximately constant even if the volume changed markedly. This could be achieved by leading the polythene tube from the cut end of the loop to a reservoir which had a comparatively large cross-sectional area. The pressure within this system was approximately 10 cm of water. This closed reservoir was then connected to a piston-recorder. In the records a downward deflection denotes a relaxation of the intestinal smooth muscles.

Stimulation of the splanchnic nerves. In order to minimize any possible leakage of the shock stimuli to adjacent nervous structures the 'Perspex' rod with the stimulating electrodes had several channels which enabled a permanent suction in the area of stimulation. This was a necessary precaution as some oozing of blood and fluid could not be avoided following the extensive dissections and the use of heparin.

A Grass model 4 B stimulator was used for stimulation. As a rule the duration of the

pulses was 2—5 msec. The voltage of the shock stimuli was 3—5. In most cases this gave a maximal response for the given frequency.

Even with training, the preparation described took about $1^1/_2$—2 hrs, but in most cases the condition of the animal was not seriously impaired. Throughout the experiment the body temperature was kept constant by use of an electric heating pad and an infra-red lamp. Blood losses were replaced with Dextran-Tyrode-solution. When preparations were completed the stimulating electrodes, the screw-clamp around the aorta and the polythene tubes for recording of blood flow and motility were all kept in fixed positions by clamps. The abdomen was closed by clips to avoid variation in temperature as well as loss of heat and humidity. No recordings were done until intestinal motility and blood flow were stabilized.

Results

The vascular responses and the effects on intestinal motility following stimulation of the preganglionic sympathetic outflow were recorded in 54 experiments. For reasons stated above frequencies of stimulation below 10/sec were preferred and the total period of stimulation was varied from 10 sec up to some minutes. These responses were then compared to the effects of adrenaline and noradrenaline injected intravenously or intra-arterially. When this comparison was done great emphasis was placed on the time-relations and also on the relative magnitude of the effects. In about half of the experiments an attempt was made to imitate the neurogenic vasoconstrictions by adjusting the screwclamp around the aorta to such an extent that an equal reduction of intestinal blood flow was obtained.

Vasoconstrictor and inhibitor responses to splanchnic nerve stimulation and adrenaline and noradrenaline

Fig. 1 *A* shows the generally recognized responses to sympathetic stimulation. The intestine was promptly inhibited and when stimulation was stopped there was a slow and gradual return of tone and rhythmicity. It should be noted that in this experiment the frequency of stimulation was 10/sec. This brought about an almost total interruption of intestinal blood flow, peripheral resistance to flow being increased some 40 times. The vascular effect was prompt and was maintained during the short period of stimulation. With the method used this record is somewhat misleading as regards the rate of development of the decrease of blood flow, since stimulation of the vasoconstrictor nerves will also reduce the 'capacity section' of the vascular bed. This will create a brief increase of outflow, to some extent masking the initial decrease of inflow caused by constriction of the 'resistance vessels'. On interrupting stimulation the vasoconstriction rapidly subsided and during the poststimulatory period there was a slight reactive hyperaemia. This increase of flow was probably likewise more prominent initially than shown by the drop-recorder, since some filling up of the vascular bed also occurred during this period, temporarily diminishing the venous outflow.

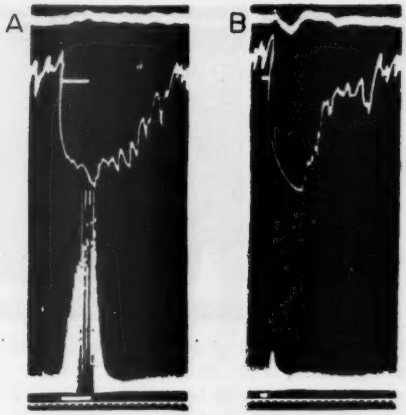

Fig. 1. Tracings of blood pressure, intestinal motility and blood flow. Time 10 sec. At *A* stimulation of the splanchnic nerves at 10/sec for 40 sec. At *B* 3 μg of adrenaline was given i. v.

Part *B* in Fig. 1 shows the response to adrenaline. The intestine was inhibited to about the same extent as that following upon splanchnic nerve stimulation. An essential difference, however, is seen in the vascular response, blood flow being decreased only to about half the initial level. The probable importance of these quantitative discrepancies will be commented upon later. Taking the time-relations into consideration it was invariably found that adrenaline and noradrenaline, reaching the intestinal loop by the blood stream in any effective dosage brought about parallel effects on motility and blood flow, inhibition and vasoconstriction commencing at the same instant. Contrary to this, however, it was generally found that the vasoconstrictor response preceded the intestinal inhibition when the sympathetic nerves were stimulated at 'physiological' frequencies. This point should be stressed when considering the nature of the inhibitory response to sympathetic nerve stimulation. Fig. 2 shows the continuation of the experiment in Fig. 1. The splanchnic nerves were now stimulated with 4/sec and the period of stimulation successively prolonged from 10 sec to 60 sec. The vascular response was still prompt and definite. Thus, 4/sec during 10 sec induced an immediate reduction of blood flow to about the same extent as did 3μg of adrenaline given intravenously in Fig. 1 *B*. On cessation of stimulation the vasoconstriction immediately started to subside While these blood flow changes occurred, proving the effectivity of the sympathetic stimulation, intestinal motility remained entirely unaffected. It is interesting to compare this fact with the marked and immediate inhibition following upon adrenaline shortly before in Fig. 1 *B*. It should here be stressed that injection of noradrenaline gave essentially similar results. *D*, *E*, and *F* of Fig. 2 show the common and important differences regarding time-relation when the vascular responses are compared with the effects on intestinal motility. At both *D* and *E* the activity of the intestinal smooth muscle cells was unin-

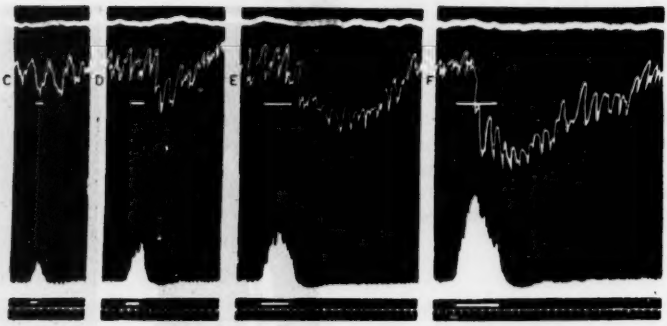

Fig. 2. Continuation of the same experiment as in Fig. 1. Stimulation of the splanchnic nerves at 4/sec during 10 (*C*), 20 (*D*), 40 (*E*), and 60 (*F*) seconds respectively. Note the prompt character of the vascular response but the long latent period and slow return of tone as regards the musculature of the intestine. Maximum inhibition coincides with a moderate reactive hyperaemia. Time 10 sec.

fluenced during the entire period of stimulation although this was continued for 20 sec and 40 sec respectively. First after the stimulation was interrupted the intestinal loop was inhibited for a considerable time. This latent period and also the prolonged character of the intestinal inhibition is in contrast to the immediate inhibition caused by blood-borne catechol-amines and is hardly in keeping with the view of a direct and specific nervous control of the intestinal smooth muscle cells, exercised by sympathetic adrenergic inhibitory nerve fibres. Injection of catechol-amines was always followed by a simultaneous appearance of the vascular and intestinal responses. Looking at *D*, *E* and *F* in Fig. 2, it is seen that the neurogenic vasoconstriction is followed by a slight reactive hyperaemia and, in fact, the inhibition of intestinal motility seems to coincide more closely with this reactive hyperaemia than with the preceding vasoconstriction.

Motility changes following passive reduction of arterial inflow

Considering the fact that sympathetic stimulation to almost any region will bring about important changes of local blood flow it is interesting to see whether variation of blood flow alone might produce any significant effects on intestinal motility. It is remarkable how few such studies there are in the literature although it must be a familiar experience to everybody working on smooth muscle cells that changes of tone and rhythmicity invariably follow even slight alterations of the local chemical environment. Fig. 3 reveals the striking parallelism in some experiments between the motility changes following sympathetic stimulation and those accompanying a mere passive reduction of arterial inflow of the same magnitude, obtained by adjusting a screw-clamp around the aorta. It should here be stressed that the intestine was completely

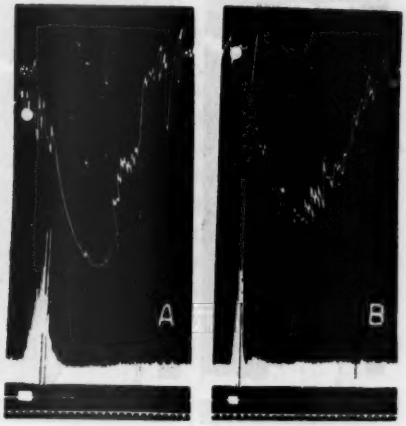

Fig. 3. At *A* stimulation of the splanchnic nerves at 4/sec during 20 sec. A similar inhibitor response was obtained at *B* when the abdominal aorta was partially occluded during 15 sec. Time 10 sec.

'decentralized' with regard to its innervation and the suprarenal glands were extirpated so that any reflex effects on the intestine, caused by the partial aortic occlusion, should be quite eliminated. At *A* in this figure the splanchnic nerves were stimulated at 4/sec, and after a latent period of some 25 sec the intestine was inhibited and it remained so for a considerable time after the stimulation was interrupted. A short but rather extensive vasoconstriction occurred immediately on the onset of stimulation but was confined to the period of stimulation. Following an equal but merely passive retardation of the blood flow the intestine showed a similar inhibitor response (*B* in Fig. 3). This inhibition, which did not occur until a latent period of some 25 sec had passed, was here preceded by a short but rather brisk contraction of the gut. The brief mechanical reduction of blood flow, which only lasted 15 sec, was obviously responsible for this inhibition. It seems logical then that the decrease in blood flow caused by the sympathetic nerve stimulation at *A* might also be made responsible for the recorded intestinal inhibition.

The results described have shown a relatively pure inhibitor response of the intestine and this was a rather consistent observation in 'good preparations'. In experiments, where for some reason intestinal tone remained low and spontaneous rhythmic activity slight, a mixed type of response was commonly seen. Fig. 4 shows such an experiment. Stimulation of the sympathetic outflow at *A* effected an immediate and marked reduction of blood flow. Despite continued stimulation the blood flow again started to increase after some 40 sec and finally reached almost the prestimulatory level. A further increase of flow followed, however, when the stimulation was interrupted. This 'escape' of the vascular response to sympathetic stimulation could probably be attributed to an accumulation of vasodilator metabolites. In this experiment intestinal tone

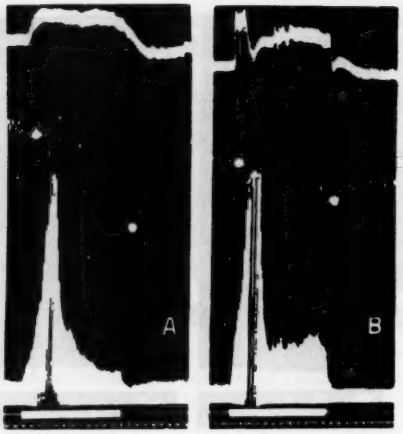

Fig. 4. Motor and inhibitor response of the small intestine from splanchnic nerve stimulation at *A* (4/sec) and from passive reduction of arterial inflow at *B*. Note the low tone and slight rhythmic activities of the intestine in this experiment. Time 10 sec.

was low and rhythmic movements were not very marked. Splanchnic nerve stimulation at 4/sec left the intestinal motility unaffected during the first 20 sec but then a rapid and rather pronounced contraction followed (the recording pen actually left the kymograph during the height of contraction). At about the same time as the vascular 'escape' started, the contraction of the gut was changed into a delayed inhibition. At *B* in Fig. 4 a similar reduction of intestinal blood flow was elicited by a partial clamping of the aorta. Although there are quantitative divergencies, the time relations and the major features of the motility response to this mechanically induced decrease of blood flow are quite similar to those effected by sympathetic stimulation.

Discussion

The present experiments have shown that the inhibitory response of the small intestine to preganglionic sympathetic stimulation differs in many respects from the responses of other autonomic neuro-effectors, which are prompt in onset. On interruption of a stimulation at physiological frequencies these neurogenic effects also disappear rapidly. In effectors such as the nictitating membrane and the dilator muscle of the iris, low-frequency stimulation might even give a series of 'single twitches'.

In contrast to these characteristics — in the present experiments represented by the prompt vasoconstriction on sympathetic stimulation — the inhibition of the small intestine often has a considerable latent period as long as the stimulation frequencies are within the physiological range. Thus, in some experiments the inhibitory response started only after 20—40 sec of continued stimulation. Its rate of development was slow and after stimulation full return

of tone and motility did not occur for the first few minutes. When, however, catechol amines were administered *via* the blood stream the inhibition occurred as rapidly as the vasoconstriction.

This delayed type of response could neither be explained by inadequate nerve stimulation nor by an insensitiveness of the intestinal smooth muscle cells to the sympathetic transmitter. This is borne out by the prominent nervous effects on the blood vessels and the fact that quite small amounts of adrenaline or noradrenaline give *via* the blood stream provoked prompt and marked inhibitions. Thus, there seems to be no acceptable reason why an adrenergic transmitter, if it were released in close contact with the intestinal muscle cells at any inhibitory fibre endings, should not have an immediate inhibitory action.

On closer examination of earlier studies delayed inhibitor responses of the intestine on sympathetic stimulation have indeed been recorded by several investigators but its functional significance appears to have been overlooked. For example, even from the illustration to BAYLISS and STARLING's original study (1899) — found in many textbooks — it appears as if the inhibition did not commence until after two complete spontaneous contraction-cycles. — More recently, GARRY and GILLESPIE (1955) systematically examined the responses to both sympathetic and parasympathetic stimulation, using an *in vitro* preparation of the rabbit's colon. Parasympathetic stimulation induced prompt motor responses at 1/sec and maximal responses around 10/sec. Sympathetic stimulation, on the other hand, was 'rarely effective' at frequencies below 5—10/sec. Only above this frequency range did inhibition occur regularly but then with a distinct latency, and it reached its maximum at frequencies about ten times higher than those required for any other autonomic neuroeffectors studied (for references see FOLKOW 1956). — Normally, discharge rates as high as 10/sec will probably only be reached under intense reflex excitation of the sympathetic nervous system, *e. g.* during extreme asphyxia. Otherwise, the sympathetic discharge range in the intact organism appears to be quite narrow, from 0 up to 6—8/sec (FOLKOW 1956). Most effectors will in fact respond promptly and markedly at frequencies well below 1/sec (CELANDER 1954) and show maximal responses around 10/sec.

The intestinal inhibition on splanchnic nerve stimulation must, to a considerable extent at least, be due to the decrease of intestinal blood flow, caused by the vasoconstrictor nerves. This is obvious when the 'neurogenic' effects are compared with those following an equal, but merely passive reduction of arterial inflow. In some experiments the two manoeuvres produced almost identical changes in intestinal motility. Since all nervous connections were cut and the adrenal glands tied off any possible central reflex interference secondary to the partial aortic occlusion was excluded. — The influence of the blood flow was recognized and discussed by BAYLISS and STARLING (1899) and they confirmed earlier observations that local ischaemia inhibited all the movements of

the intestine. But, though they agreed that the intestinal inhibition produced by splanchnic stimulation was similar to that obtained by obstructing the aorta, they concluded that the 'neurogenic' inhibition must have been due to another mechanism, and therefore they proposed the inhibitory sympathetic fibres. Their main argument was the fact that an inhibitory response occurred on splanchnic stimulation even when the circulation was stopped by 'cutting out the heart'. This might appear to be a crucial test and it certainly proves that there is another factor beside the change in blood flow. However, in BAYLISS and STARLING's study, as in practically all previous investigations, the stimulation frequencies applied were far above the physiological range, generally 20 per second or more. At these frequencies, also used by FINKLEMAN (1930) in an *in vitro* preparation, it is known that a considerable 'overflow' of the adrenergic transmitter from the vasoconstrictor nerve endings takes place. At physiological frequencies below ten impulses per second, on the other hand, the escape of the adrenergic transmitter is too small to affect non-innervated cells significantly (CELANDER 1954, BROWN and GILLESPIE 1957, BROWN, DAVIES and GILLESPIE 1958) though minute amounts appear in the urine (EULER 1956). No transmitter substance can, for instance, be detected in the venous outflow when the splenic nerves are stimulated at frequencies below 10/sec; at this frequency it begins to appear and increases rapidly to a maximum at a rate around 30/sec. The explanation seems to be that at frequencies of 10/sec and higher the interval between the nerve impulses is too short to enable a complete local inactivation of the transmitter, hence it begins to diffuse to the extracellular fluid and to the blood stream (FOLKOW 1952, CELANDER 1954, BROWN and GILLESPIE 1957). Considering the normal range of discharge within the sympathetic nervous system an overflow of this order should be considered as an experimental artefact, as such high frequencies never seem to occur in the intact organism. Since an overflow has been shown to take place within the intestine at such high rates (BROWN, DAVIES and GILLESPIE 1958), the vasoconstrictor fibre transmitter will diffuse from the site of release and secondarily affect the adjacent intestinal smooth muscle cells. This would explain the failure of GARRY and GILLESPIE (1955) to obtain inhibitor responses *in vitro* at frequencies below 5—10/sec. It would also explain why they did obtain inhibitory responses at higher stimulation rates. In my opinion, the frequency-response characteristics of the intestinal inhibition on sympathetic stimulation in GARRY and GILLESPIE's study can rather be taken as further evidence against any centrally controlled, inhibitory fibres to the intestinal muscles.

Summing up then, the inhibitory response of the intestine on splanchnic stimulation can be satisfactorily explained as being due to the combined effect of the reduced blood flow following vasoconstrictor fibre activation and a minute 'overflow' of the constrictor fibre mediator. As this 'overflow' is known to increase rapidly when supra-physiological frequencies are applied, it will under such circumstances be able to induce powerful inhibitory responses on

the intestinal smooth muscle cells. Equally, similar mechanisms may explain the inhibition of the uterus and bronchi in response to sympathetic stimulation.

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Adenosinephosphates in the Rat Adrenal Medulla

1. Adrenal Medulla in Minimal Secretory Activity

By

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Abstract

HILLARP, N.-Å., B. JÖNSSON and G. THIEME. Adenosinephosphates in the rat adrenal medulla. 1. The adrenal medulla in minimal secretory activity. *Acta physiol. scand.* 1959. *47*. 310—319.—A method based on high voltage paper electrophoresis has been devised permitting simultaneous determination of the adenosinephosphates and catechol amines present in four rat adrenal medullas. Practically all of the ATP (14 μmoles/g wet weight) in the medulla seems to be bound to the amine granules in the living cell. Besides ATP, the medulla contains quite exceptional contents of AMP and ADP (1.5 and 5.3 μmoles/g). The significance of these nucleotides is discussed. No change in the adenosinephosphate pattern was found after denervation of the medulla.

Previous studies (for references see HILLARP and THIEME 1959) suggested that analysis of the changes in the adenosinephosphates and catechol amines in the adrenal medulla under conditions of varied secretory activity might be of value in elucidating the storage and release mechanisms of the amines. A convenient method for the simultaneous determination of these compounds in the rat medulla was therefore worked out. This paper is concerned with the adrenal medulla in minimal secretory activity.

Methods

Adult male rats (350—400 g) from a common stock were used. To secure experimental conditions as identical as possible to those used in another study (to be reported in a forthcoming paper) the animals were deprived of food but not water for 20 hours before being killed. They were killed by a blow on the head and decapitation. The adrenals were removed without delay and chilled with ice. To minimize the interval between removal and extraction, the medulla was dissected out of only two or at the most four animals at a time.

Extraction procedure: The glands were cut into two halves and the medulla was cut out with a pair of fine scissors. The medulla is firmer than the cortical tissue and in skilled hands it can easily be removed entirely and rapidly. The medulla was placed on a white, hard fairly thick paper and any small adherent pieces of cortical tissue were removed with a microneedle with a bent tip. It was then transferred to a small conical centrifuge tube in an ice bath. After dissection of the glands the tube was transferred to boiling water for two minutes and then chilled again. The medullary pieces were immediately crushed in 100 μl of 60 % ethanol, the crushing being repeated several times during 15 min. The tubes were kept at — 25° over night.

In all experiments the medullary tissue from four glands was used to each extract.

Paper electrophoresis: The tubes containing the extract was centrifuged and 50 μl transferred to a Whatman no. 1 paper, previously washed with N HCl and Versene (pH 8). The components were separated by high voltage electrophoresis (3 kV for 45 min, 55 V/cm, acetic acid-acetate buffer of ionic strength 0.1 and pH 4.15), with the paper in carbon tetrachloride at about + 10°. The ionophoresis technique was adopted from BERGKVIST (1957, 1958).

The paper was dried at room temperature. Before the paper was dry the catechol amine containing region of the paper was exposed to HCl fumes to prevent oxidation of the amines. The adenosinephosphates and amines were located in ultraviolet light from a high pressure mercury lamp with a Schott UG 5 filter and by making a contact print (Philips TUV, 15 W lamp; peak emission at 254 mμ). With the latter method adenosinemonophosphate may be detected in amounts below one μg. The spots were cut and eluted with 1.0 ml of 0.1 N HCl, and the ultraviolet spectra of the eluates were taken. Blanks were obtained by cutting out pieces of the paper adjacent to the spots (at the same distance from the base line) of exactly the same size and shape as used for the spots. This is necessary if the measurements are to be reliable.

The same reference substances and the same molecular extinction coefficients were used as in a previous study (HILLARP 1958 b). In the calculation of the total catechol amine content of the extracts a molecular weight of 181 was used since noradrenaline represents about 15 % of the total amines (HÖKFELT 1951).

Paper chromatography: Eluates from adenosinephosphate spots were pooled and the phosphates were hydrolyzed in N HCl at + 100° for one hour. After concentration *in vacuo* the solution was examined by paper chromatography as described in a previous paper (HILLARP and THIEME 1959). Chromatography of ethanol extracts of whole medulla was performed using systems I and III as described in Circular OR-10 (1956) from the Pabst Laboratories.

Cell fractionation: The medullary tissue from 8 glands at a time was homogenized in 8 ml of 0.3 M sucrose and fractionated by centrifugation (cf. HILLARP and THIEME 1959). A low-speed sediment (800 × g for 6 min.), containing connective tissue, unbroken cells, nuclei etc., and a high-speed sediment (38,000 × g for 60 min.), containing all amine granules, mitochondria and probably much microsomal material, were isolated. They were extracted and analyzed as described above. The final super-

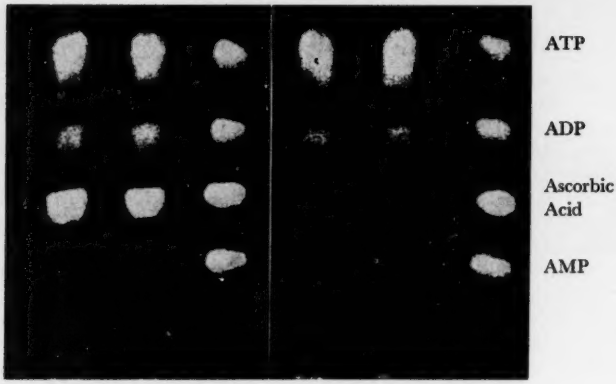

Fig. 1. Paper electrophoresis of extracts of whole rat medulla (A) and of the total granule fraction (C) from rat medulla.
B: standard containing ATP, ADP, AMP and ascorbic acid (about 10 μg of each).
Contact print by ultraviolet light.

natant ("soluble" cytoplasm, microsomal material and other small particles) was treated with trichloro acetic acid. The protein-free extract was neutralized and the catechol amines were taken up on a small cation exchange column (Dowex 50, 150 to 300 mesh, 16 × 5 mm, pH 6). The amines were eluted with N HCl and determined by ultraviolet absorption.

Pure amine granules were obtained by density gradient centrifugation as described previously (HILLARP and THIEME 1959). The medullary tissue from 18 glands was used in each experiment.

Denervation of the adrenal medulla: By an intraabdominal approach the great splanchnic nerve was cut on the left side immediately cranial to its exit from the thorax. Such division totally denervates the medulla of the rat (HILLARP 1946). The animals were killed two weeks after operation. The denervated medullas from four animals were used for each extract, the right glands of the same animals serving as a control.

Results

Evaluation of the method for determination of adenosinephosphates and catechol amines

The electrophoresis method used gives a good separation of the adenosinephosphates (AMP, ADP and ATP) present in the adrenal medulla (Fig. 1). No or only small amounts of interfering substances were present in the eluates of the spots, so that almost pure ultraviolet spectra were obtained. This holds good for the catechol amines, too. The recovery of pure substances was satisfactory: 95—105 % for the adenosinephosphates in amounts down to about 3 μg and for adrenaline down to about 7 μg. Determination of smaller amounts

Table I. Content of catechol amines and adenosinephosphates in the rat adrenal medulla (from 4 glands)

		Amines µmoles	ATP µmoles	ADP µmoles	AMP µmoles	µmoles per µmole Amines		
						ATP	ADP	AMP
Left, denervated glands	I	0.340	0.0750	0.0225	0.0088	0.220	0.066	0.026
	II	0.355	0.0930	0.0295	0.0074	0.260	0.083	0.021
	III	0.340	0.0825	0.0310	0.0086	0.240	0.091	0.025
	IV	0.350	0.0825	0.0325	0.0093	0.235	0.093	0.027
	V	0.360	0.0855	0.0300	0.0082	0.235	0.083	0.023
	Mean	0.349	0.0837	0.0291	0.0085	0.238	0.083	0.024
Right, normal glands	I	0.380	0.0825	0.0245	0.0081	0.220	0.065	0.021
	II	0.390	0.0965	0.0365	0.0100	0.245	0.094	0.026
	III	0.345	0.0890	0.0310	0.0087	0.260	0.090	0.025
	IV	0.365	0.0820	0.0340	0.0100	0.225	0.093	0.027
	V	0.370	0.0875	0.0305	0.0089	0.235	0.083	0.024
	Mean	0.370	0.0875	0.0313	0.0091	0.237	0.085	0.025
Normal glands	VI	0.350	0.0780	0.0350	0.0098	0.220	0.100	0.028
	VII	0.380	0.0830	0.0315	0.0087	0.220	0.083	0.023
	VIII	0.345	0.0790	0.0300	0.0100	0.230	0.087	0.029
Mean of all normal glands		0.366	0.0847	0.0316	0.0093	0.232	0.087	0.025

is possible but then involves a larger error. Repeated determinations on the same extract (cat and cow adrenal medulla) showed a single determination not to deviate from the mean by more than 6 %.

The highly active ATPase and other enzymes involving adenosinephosphates in the adrenal medulla (HILLARP 1958 a) were inactivated by the heating and the 60 % ethanol used for extraction. This was found in experiments with extracts from cow medulla. No changes in the adenosinephosphates present were observed when the extracts containing the crushed medullary tissue were kept at room temperature for two hours. The heating also served two other purposes: to facilitate the crushing of the tissue and to decrease the solubility of the proteins.

That the extraction and electrophoresis procedures do not cause any breakdown of ATP is clear from the fact (see below) that only ATP but no ADP or AMP were detected in the pure amine granules isolated from rat medulla.

A slight contamination of the medullary extracts with cortical tissue is unavoidable. As found on electrophoresis of cortical extracts, this tissue — as compared with the medulla — has such a low content of adenosinephosphates, however, that no significant amounts of these compounds would be added to the medullary extracts by the contaminating cortical cells.

Table II. Content of catechol amines and adenosinephosphates in the rat adrenal medulla per g wet weight

	Per g wet weight	
	mg	μmoles
Amines	11.0	61
ATP	7.1	14
ADP	2.3	5.3
AMP	0.52	1.5

From the data in Table I and in a previous paper (CARLSSON and HILLARP 1956), it can be calculated that the four medullas used to each extraction with 100 μl of ethanol weighed about 6 mg. The increase in the volume by the addition of the tissue water was thus so small that it was neglected. This seems all the more justified since the error introduced must be fairly constant.

The catechol amine content in normal glands (Table I) was found to be somewhat lower than expected (cf. HÖKFELT 1951). This may be due to a strain difference or to losses on dissection of the medullas. Table I shows however, that the values found are in good agreement. If losses occur, they must thus be fairly constant.

Adenosinephosphates in the normal adrenal medulla

Only four spots absorbing at 254 mμ were detected on ionophoresis of extracts from whole medulla (Fig. 1). One of them was found not to contain any nucleotide and the component had properties apparently identical with those of ascorbic acid. The other three components showed the same mobility on paper electrophoresis and chromatography as AMP, ADP and ATP, respectively, and ultraviolet spectra practically identical with those of these nucleotides. After hydrolysis practically only adenine was found. The methods used seem to justify the assumption that they are identical with these phosphates. Other nucleotides and substances may be present in the ionophoresis spots, but probably not in sufficient amounts seriously to interfere with the determinations of the adenosinephosphates. There is little doubt that the main compound is ATP since this is the principal nucleotide present in the amine granules and judging from the amine content of the medulla the amounts found were such as might be expected (cf. HILLARP and THIEME 1959). Furthermore, the values determined by our method are in fairly good agreement with those obtained by SCHÜMANN (1958) using the firefly method.

The content of the adenosinephosphates can be calculated (Table II) from

Table III. Catechol amines and adenosinephosphates in the total granule fraction (38,000 × g for 60 min.) and in pure amine granules obtained by density gradient centrifugation. The values in experiments I—IV were corrected for the degree of homogenization so that the total granule fraction in each experiment represents the whole fraction from four glands at complete homogenization. In this way the values are directly comparable to those in Table I

Granule Fraction	Total: μmoles				μmoles per μmole Amines		
	Amines	ATP	ADP	AMP	ATP	ADP	AMP
I	0.345	0.0770	0.0165	0.0084	0.225	0.047	0.024
Total .. II	0.330	0.0795	0.0170	0.0087	0.240	0.052	0.026
Granule III	0.335	0.0780	0.0180	0.0100	0.235	0.054	0.030
Fraction IV	0.360	0.0890	0.0195	0.0096	0.245	0.054	0.027
Mean	0.342	0.0809	0.0177	0.0092	0.236	0.052	0.027
Pure.... V	0.255	0.0625	< 0.004	< 0.004	0.245		
Amine Granules... VI	0.290	0.0730	< 0.004	< 0.004	0.250		

the catechol amine content per g medulla (CARLSSON and HILLARP 1956, and unpublished results). This clearly shows the high concentrations present.

Adrenal medullas were homogenized and fractionated in an attempt to locate the adenosinephosphates. As it is technically difficult to determine small amounts of these substances in the final supernatant containing the "soluble" cytoplasmic fraction, only the low-speed sediment and the granule fractions were analyzed (Table III). In the pure amine granules practically only ATP was present, the amount being about equivalent to that of the catechol amines. The total granule fraction (all amine granules and mitochondria, probably much microsomal material and other particles) contained most — if not all — of the ATP and AMP in the medulla and more than half of the ADP, as seen from the data in Tables I and III (see also Fig. 1). The amounts of adenosinephosphates in the low-speed sediment containing inter alia the cell nuclei were too small to be determined and were probably satisfactorily accounted for by the unbroken cells present (7 to 10 % as calculated from the distribution of the catechol amines).

Adenosinephosphates in the denervated adrenal medulla

In a series of rats (Table I) the left denervated medullas were compared with the right non-denervated glands. The absolute amounts of catechol amines and adenosinephosphates were somewhat lower in the denervated glands, but their mutual proportions were the same. Thus denervation does not produce any significant change in the adenosinephosphate pattern in the medullary cells.

Discussion

In all animals examined in previous studies (cf. HILLARP and THIEME 1959) the amine granules of the adrenal medulla were found to contain ATP, ADP and AMP in varying proportions but always in amounts practivally equivalent with the stored amines. As shown in the present paper, the rat is no exception in this last respect. There is, however, a peculiar difference between the amine granules of this animal and those of others, in that the rat granules store practically only ATP together with the amines.

Cell fractionations show (Table III) that 90 to 92 % of the amines in the rat medulla are recoverable in the large granule fraction, a finding in agreement with the result obtained in a previous study (CARLSSON and HILLARP 1956). Since homogenization damages some of the amine granules with consequent release of amine (HILLARP, LAGERSTEDT and NILSON 1953), at least 90 % and probably more of the amines must be bound to the granules in the living cell. If all the amine granules contain ATP and amines in equivalent amounts (as found for the fraction of pure granules; Table III), it may thus be calculated that practically all of the ATP actually determined in the medulla must be bound to the amine granules. This is in agreement with the observation that most — if not all — ATP was recovered in the total granule fraction (compare data in Table I and III). There is an uncertainty in this calculation, however, since it is not known whether the molar ratio ATP: amines is the same in all amine granules.

Since 8 to 10 % of the catechol amines in the homogenized medulla were not recovered in the granule fraction there is a possibility that these amines together with a corresponding amount of ATP have been released from their granules after death. This might give an accumulation of ADP and AMP in the cells. It may be calculated from the data in Table I, however, that only a small portion (at most about 20 %) of the ADP and AMP found in the whole cells can be derived from ATP broken down in this way. The living cell may thus contain either large amounts of ADP and AMP or a very labile ATP fraction not belonging to the ATP stored together with amines and that is split before there is time to chill and extract the glands. The latter alternative does not seem so likely, since the animals were killed and the glands chilled within a minute. Furthermore, denervation of the medulla did not result in any change in the adenosinephosphate pattern.

The contents of free nucleotides in several rat tissues have been determined by SAUKKONEN (1956). In the nucleotide-rich liver and kidney tissue the adenosine-5-phosphates represent about 70 % of the total free nucleotides, and ADP and AMP are the largest fractions. But the sum of these fractions is small (1.66 and 1.35 μmoles per g wet weight) compared with the content in the rat medulla (6.8 μmoles; Table II). The fact alone that the normal medullary cells with their low amine secreting and synthetizing activity have such a high

content of ADP and AMP supports the assumption that these compounds not only participate in the general metabolism but in reactions directly serving the specific metabolism of the medullary cells.

It has not been possible definitely to determine where the large amounts of ADP and AMP are localized in the cells. Most, if not all, of the AMP and more than half of the ADP were recovered in the total granule fraction and are thus probably in some way bound to structures in the living cell. The remaining ADP may be contained in the cell structures constituting the microsomal material of homogenates, or it may have been released from its site in the living cell on cell fractionation. It does not seem likely that it exists in true solution, since the large granules possess readily accessible adenylate kinase activities (HILLARP 1958 a).

If the assumption is correct that all the amine granules have the same composition as regards adenosinephosphates (Table III), it must be concluded that the bulk of ADP and AMP of the rat medulla is present in cell structures other than the amine granules. Considerations on the medullary content of adenosinephosphates in relation to that of catechol amines seem to support this view. It may be calculated from the data in Table I that the adrenal medulla contains considerably more adenosinephosphates than are necessary for the storing of the amine. If the ADP and AMP present are thought to be stored together with amines, about 25 % of the ATP must be excluded from the amine storage. If, on the other hand, most of the ATP serves this purpose, most of the ADP and AMP must have other functions. The significance of these calculations is best realized when considering the quite exceptional amounts of adenosinephosphates present in the rat medulla (see above).

There are, however, arguments in favour of the view that the adenosinephosphates to the main part belong to the amine granules in the living cell despite the considerations above. It is hard to imagine that the general cytoplasmic structures (mitochondria etc.) of the medullary cell should hold quite exceptional amounts of these nucleotides, especially since this cell contains only a small number of mitochondria (HILLARP, HÖKFELT and NILSON 1954; see also CARLSSON, HILLARP and HÖKFELT 1957). Furthermore, no special structures other than the amine granules seem to be present (see electron micrographs in WETZSTEIN 1957).

A reasonable explanation for the presence of the large amounts of ADP and AMP might be that these phosphates represent a reserve material kept in the granules for the amine storage. The continuous process of amine storing may start with an incorporation of AMP and ADP, so that many granules at any given moment contain adenosinephosphates in excess of amines. That the pure amine granules isolated by density gradient centrifugation were found to have equivalent amounts of amines and adenosinephosphates may readily be explained on the basis that the granules with a maximal store of amines also have the highest density. This is supported by the observation of SCHÜMANN

(1958) that the density of the granules decreases when they are depleted of amines on stimulation of the medulla.

It might be argued that the ADP and AMP present in the medulla are merely accumulated split products of amine granule ATP released during amine secretion somé time before death. The fact that the denervated medulla has the same adenosinephosphate pattern as the normal gland speaks definitely against this assumption. Denervation considerably reduces the resting secretion of the medulla (DUNÉR 1953) but the content of catechol amines does not undergo any definite changes (HÖKFELT and MCLEAN 1950), which was confirmed in our experiments. Further, no accumulation of adenine compounds was found in the cytoplasm of medullary cells (sheep) stimulated to secretion via their autonomic nerves (CARLSSON, HILLARP and HÖKFELT 1957). This will be more adequately shown in a forthcoming study on rats examined in different phases of amine secretion and resynthesis.

Summary

1. A rapid and convenient method has been worked out permitting simultaneous determination of the adenosinephosphates and catechol amines present in the medullary tissue (about 6 mg wet weight) from four rat adrenal glands. After short heating at + 100°, the tissue was extracted with 100 μl of 60 % ethanol. The compounds present in half of the extract were separated by high voltage paper electrophoresis and, after elution, determined by ultraviolet absorption.

2. By use of this method the contents of AMP, ADP, ATP and catechol amines in the normal and denervated medulla and their intracellular distribution have been studied.

3. Pure amine granules isolated by density gradient centrifugation contain ATP and amines in about equivalent amounts, but at most only small amounts of AMP and ADP. Practically all of the ATP (14 μmoles/g wet weight) in the medulla seems to be bound to the amine granules in the living cell. At least 90 % of the catechol amines are bound in this way.

4. The rat adrenal medulla contains — as compared with other tissues — quite exceptional contents of AMP and ADP (1.5 and 5.3 μmoles, respectively, per g wet weight). It is possible that these nucleotides in the living cell are present in amine granules that have not stored amines to their maximal capacity.

5. The denervated adrenal medulla showed the same adenosinephosphate pattern as the innervated medulla.

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Influence of Exogenous Thyrotropic Hormone on Regeneration of Thymus and Lymph-Node Tissue after Cortisone-Induced Involution

By

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Abstract

Barnholdt-von Euler, U., L. Gyllensten, and U. Tidén. Influence of exogenous thyrotropic hormone on regeneration of thymus and lymph-node tissue after cortisone-induced involution. *Acta physiol. scand.* 1959. *47*. 320—327. — The growth-stimulating influence of exogenous thyrotropic hormone on the lymph-node tissue of young guinea pigs was found to be enhanced during cortisone-induced involution. No such effect was found as regards the thymus tissue, nor did pretreatment with cortisone-acetate enhance the thyroid stimulation by thyrotropic hormone (thyroid weight and mean acinar cell height). It is concluded, that the increased stimulation of the lymphatic tissue in experimental hyperthyroidism after previous administration of cortisone-acetate is dependent neither on increased elimination of the cortisone-acetate nor on increased thyroid stimulation. It is probably to be ascribed to an increased tendency to proliferation localized directly to the lymphatic tissue.

It is a well established fact that an excess of thyroid hormone can, when its concentration is suitable, produce hyperplasia of the thymus and lymphatic tissue. This has been shown by a number of clinical observations in man, as well as by experimental studies in various animal species (for references, see Hammar 1936, Grégoire 1942, Gyllensten 1953, Ernström and Gyllensten 1959).

The extent to which the thyroid gland participates in regulation of normal growth of the aforementioned tissues is unknown. On the other hand, various experimental studies have shown that this gland partakes in regenerative processes following damage to the thymus or lymphatic tissue. As early as 1942, GRÉGOIRE demonstrated that thyrotropin stimulates regeneration after roentgenologic involution of the thymus.

Moreover, it has been shown by one of us (GYLLENSTEN 1953) that resection of the thymus in growing rats leads to hyperplasia of the lymphatic tissue. In addition, the postoperative growth of this tissue is associated with transient, slight hyperactivity of the thyroid.

BODLUND and GYLLENSTEN (1954) have shown that prolonged administration of cortisone acetate to young guinea pigs leads to an increased uptake of inorganic P^{32}-labelled phosphate per unit weight in the greatly involuted thymus and lymph-node tissue, as well as in the spleen. Such increased P^{32} uptake, localized principally to the protein-bound phosphate and the main part to its nucleoprotein-bound fraction, is a criterion of increased cytopoietic activity in the aforementioned organs (HEVESY and OTTESEN 1943, ANDREASEN and OTTESEN 1944, 1945, GYLLENSTEN and RINGERTZ 1954, GYLLENSTEN, RINGERTZ and RINGERTZ 1956). Thus, an increased new formation of cells probably took place in these tissues during the continuous cortisone therapy, despite a decrease in weight of the organs. There was concurrent thyroid stimulation, judged by the weight of the gland, mean acinar cell height and phosphate uptake, even though activation of the thyroid by thyrotropic hormone administered was less than normal. These results indicate that the continuous cortisone administration elicited homeostatic mechanisms affecting the pituitary-thyroid complex, their action stimulating regeneration in the involuted thymo-lymphatic tissue.

The object of the investigation reported in the present paper was to study the influence of thyroid activation on thymo-lymphatic tissue after a single dose of cortisone.

Material and Methods

Young guinea pigs, weighing 150—250 g, were divided into 4 groups of equal size, so that the groups were similar with respect to weight and sex distribution of the animals. These groups are denoted in the following as O, C, TSH and C+TSH.

Group O consisted of untreated controls. Group C consisted of animals given 15 mg of cortisone acetate (Upjohn) i.p. and killed 6 days later. The animals in group TSH were given 1 guinea pig unit of thyroid-stimulating hormone (Ambinon, Pharmacia) s.c. per day on three consecutive days, and were killed 1 day after the last injection. The animals in group C+TSH received cortisone acetate in the same way as those in group C, and thyroid-stimulating hormone in the same dose as those in group TSH on the 3rd, 4th and 5th day after administration of cortisone; they were killed 1 day after the last injection of thyrotropin.

Before starting the experiments, the animals were grouped by pairs, so that each

animal in group C had a partner of the same sex and of as nearly as possible the same size in group O. In the same way, each animal in group C+TSH was assigned a partner in group TSH. The statistical comparisons between the groups, with respect to the weight of the organs examined and thyroid cell height, were based on the mean value of the differences between the paired animals, and not on differences between the mean values for the different groups. The former method of calculation has been found to give less scattering than the latter (GYLLENSTEN 1953).

Dissection of the thyroid gland and thymus, as well as of the cervical and inguinal lymph nodes was performed quantitatively, using the technique described by GYLLENSTEN (1953). In guinea pigs, a good correlation exists between the weight of these lymph nodes and the total mass of lymph-node tissue (GYLLENSTEN). In the calculations, the weight of the organs was put in relation to the body weight of the animal, *i. e.*, as mg of organ tissue per g of body weight.

The thyroids were fixed in 10 per cent formaldehyde, embedded in paraffin and cut into at least 10 sections 5 μ thick through the central part of the gland. The specimens were stained with iron chloride and haematoxylin according to HÄGGQVIST (regressively). After this, 25 acinar cells per thyroid, taken at random, were measured in a microcamera at a magnification of 1,000 $\times$. The examiner did not know from which group of animals the specimen in question was taken.

Before the actual experiments were started, a preliminary experiment was made to determine the time course of thymo-lymphatic involution in young guinea pigs after a single dose of 15 mg of cortisone acetate. This preparation was given to 44 guinea pigs of the same weight as the animals in the aforementioned groups. They were then killed in groups of 4, the first group after 12 hours, and one group daily after 1—10 days. Six animals were untreated controls. The weight of the thymus and of the cervical and inguinal lymph nodes, as well as of the spleen, was determined. In addition, the uptake of radioactive, inorganic phosphate was measured in these organs (except the spleen), as well as in the thyroid gland and in the adipose and muscle tissue, using the method described by BODLUND and GYLLENSTEN.

This preliminary experiment showed a continuous, rectilinear decrease in weight of the thymus, starting on day 1 after the injection and continuing until day 10, when the relative weight of the thymus per unit body weight had fallen to 43 per cent. No definite effect on the weight of the lymph nodes, spleen or thyroid could be demonstrated. On the other hand, the thyroid gland showed a slight decrease in the relative phosphate uptake per unit weight, as compared to that in the adipose and muscle tissue. This decrease was not, however, significant ($p > 0.05$). No difference could be demonstrated between the cortisone-treated animals and the controls with respect to the phosphate uptake in the other organs and tissues.

Results

The mean values of the relative weight of the organs and of thyroid cell height in the different groups are recorded in Table I. The results of statistical analysis of the differences between the groups, based on comparisons between the paired animals, are shown in Table II.

It is evident from Table I and II that administration of cortisone acetate alone produced a decrease in weight of the thymus and lymph-node tissue amounting to about 13—18 per cent, this decrease being highly significant. On the other hand, it resulted in no demonstrable change in thyroid activity.

Table I. Relative weight of organs (mg/g body weight) and mean thyroid cell height (μ) in untreated guinea pigs (group O), cortisone-treated (group C), thyrotropin-treated (group TSH) and in guinea pigs treated first with cortisone and then with thyrotropin (group C + TSH). Ln = lymph nodes

Group	Thyroid		Relative weight of			No. of animals
	Rel. weight	Cell height	Thymus	Cervical Ln	Inguinal Ln	
O	0.184	5.84	1.026	0.696	0.140	30
C	0.182	5.89	0.836	0.585	0.108	29
TSH	0.289	10.26	1.159	0.659	0.212	30
C + TSH	0.280	10.03	0.896	0.678	0.192	30

Table II. Percentage differences between relative weight of organs (mg/g body weight) and thyroid cell height (μ) in the animals in group C and their paired controls in group O, as well as in the animals in group C + TSH and their paired controls in group TSH. For treatment of the animals in the respective groups, see legend to Table I. Conventional statistical abbreviations are used. Degrees of significance: — = not significant ($p > 0.05$), ++ = significant ($p < 0.01$), +++ = highly significant ($p < 0.001$)

	Thyroid		Relative weight of		
	Rel. weight	Cell height	Thymus	Cervical Ln	Inguinal Ln
Difference in group C compared to group O	— 0.31	+ 1.10	— 17.79	— 12.86	— 17.10
Standard error	1.82	1.78	2.58	2.68	6.18
df	28	28	28	28	28
t	— 0.17	0.62	— 6.88	— 4.80	— 2.77
Significance	—	—	+++	+++	++
Difference in group C + + TSH compared to group TSH	— 2.40	— 2.52	— 21.77	+ 3.77	— 2.03
Standard error	1.71	0.81	2.80	2.72	3.48
df	29	29	29	29	29
t	— 1.41	— 3.07	— 7.79	1.38	— 0.58
Significance	—	++	+++	—	—

It can be inferred from Table I that when thyrotropic hormone only was given, it produced marked thyroid stimulation. The increase in weight of the thyroid and in its mean cell height amounted to 57 and 76 per cent, respectively, and was highly significant in both cases. (The relevant statistical calculations are not apparent from the table.) Moreover, thyrotropic hormone alone produced an increase in the relative weight of the thymus and inguinal lymph nodes of 13 and 52 per cent, respectively, but a decrease of 5 per cent

in the relative weight of the cervical lymph nodes. The differences are, in the order given, almost significant, significant and not significant ($p < 0.05$, $p < 0.01$ and $p > 0.05$).

Table I also shows that administration of thyrotropic hormone after previous cortisone treatment arrested the cortisone-induced involution of the lymph nodes. It brought about restitution of their weight, so that no involution was any longer demonstrable in comparison to untreated animals. This effect of the thyrotropic hormone was confined to the lymph nodes. Involution of the thymus continued to almost the same degree as in the animals given cortisone only.

A better idea of the influence of the thyrotropic hormone in cortisone-treated animals is obtained from a comparison between groups C+TSH and TSH according to Table II. This comparison shows that, after thyrotropin administration, the relative weight of the lymph-node tissue was the same, irrespectively of whether or not the animals had been pretreated with cortisone. As stated earlier (*cf.* Table II), administration of cortisone alone (group C) led to marked involution of the lymph nodes, as compared to untreated controls (group O). This difference between lymph-node involution after cortisone administration only and after cortisone combined with thyrotropin can be calculated from Table II. It amounts to 16.63 per cent for the cervical lymph nodes and to 15.07 per cent for the inguinal, the standard error being 3.82 and 7.03, respectively, and the corresponding t values 4.35 and 2.14. The difference is highly significant for the cervical lymph nodes and almost significant for the inguinal ($p < 0.001$ and $p < 0.05$, respectively).

Similar calculations of the thymus involution after cortisone administration combined with thyrotropin and of the latter only (groups C+TSH and TSH) can be made on the basis of the figures in Table II. They show highly significant involution of about the same degree as after cortisone therapy alone (group C compared to group O), the figures being 21.77 and 17.79 per cent, respectively. The difference, which is not significant, amounts to 3.98 per cent; $t = 1.04$.

Slight inhibition of thyroid activation by exogenous thyrotropin after previous administration of cortisone can be inferred from Table II (group C+TSH compared to group TSH). As far as the increase in mean cell height after thyrotropin is concerned, this inhibition is significant ($t = 3.07$). After administration of cortisone alone (group C compared to group O), no thyroid inhibition was demonstrable. The difference between the thyroid reaction to cortisone alone and to cortisone combined with thyrotropin is not, however, significant, evaluated on the basis either of weight or of cell height (t value of the difference = 0.84 and 1.88, respectively).

An interesting feature is that thyrotropic hormone was able to arrest the cortisone-induced lymph-node involution, despite the fact that no increased thyroid stimulation occurred, but rather a slight inhibition of stimulation.

It is also noteworthy that involution of the thymus was, on the contrary, uninfluenced by administration of thyrotropic hormone; it continued to take place, in contrast to that of the lymphatic tissue.

Discussion

The involution of the thymus and lymphatic tissue observed after administration of cortisone acetate is in conformity with the already established facts. Greater involution of the thymus than of the lymphatic tissue has also been described earlier.

The influence of adrenocortical steroids on the state of activity of the thyroid gland is more irregular, even if most authors have stated that cortisone has an inhibitory effect (for references and data, see GRÉGOIRE 1942, MONEY *et al.* 1951, D'ANGELO *et al.* 1953, BODLUND and GYLLENSTEN 1954, BROWN-GRANT *et al.* 1954, GOLDENBERG *et al.* 1958, KRÜSKEMPER and DOERING 1959). In this respect, there may be considerable differences between various corticosteroids, as well as between different animal species. Moreover, the duration of treatment is of importance. For example, BODLUND and GYLLENSTEN noted stimulation of thyroid activity in guinea pigs after lengthy administration of cortisone acetate. This agrees with GERWING's finding (1958) that chronic stress produced thyroid activation in guinea pigs and rats. The guinea pigs nevertheless reacted differently to the rats, in that an initial period of decreased thyroid activity appeared in the latter, but was lacking in the guinea pigs (*cf.* GERWING *et al.* 1958).

The mechanism of action of cortisone and other steroids on thyroid activity is unknown. Three main possibilities are open, *i. e.*, an effect on (1) the pituitary production of thyrotropin, (2) the excretion or metabolism of thyrotropin, thyroxin or iodine, and (3) a direct action on the thyroid gland (for discussion, see the publications listed above and INGBAR 1953, D'ANGELO 1955, INGBAR and FREINKEL 1956, BASTENIE and ERMANS 1958, BECK 1958). The conditions are further complicated by the facts that the thyroid hormone influences the functions of the adrenal cortex (*e. g.* LEVIN and DAUGHADAY 1955), and that the thymus and lymphatic tissue possibly affect the metabolism of thyrotropin (RAWSON and MONEY 1949).

In the present investigation, no definite effect on the thyroid gland could be noted after a single dose of cortisone acetate. On the other hand, pretreatment with cortisone resulted in a slight inhibition of activation of the gland by exogenous thyrotropin. The same effect has been demonstrated earlier after lengthy cortisone therapy in guinea pigs (BODLUND and GYLLENSTEN). Similar results in rats have been reported by KRÜSKEMPER and DOERING. There is thus a difference between the thyroidal effect of cortisone on intact animals with endogenous thyrotropin only, and on those given exogenous thyrotropin as well. This seems to indicate that cortisone acts directly on the thyroid

gland, and not on the pituitary production of thyrotropin, or on the metabolism of thyrotropin, thyroxin or iodine.

The main result of the present study is demonstration of the fact that exogenous thyrotropin produced a greater relative increase in weight of the lymphatic tissue after previous cortisone therapy than without it, so that no cortisone-induced involution was any longer demonstrable. This can not be ascribed to the thyrotropin having increased the breakdown or excretion of the cortisone acetate administered, since involution of the thymus continued. *Nor* can it have been due to greater activation of the thyroid after cortisone administration, so that it produced more thyroid hormone which, in turn, stimulated lymphatic growth, with resulting compensation of the cortisone-induced involution. For, activation of the thyroid gland was, on the contrary, less marked when thyrotropin stimulation was preceded by cortisone administration.

It seems most probable that the increased reaction of the cortisone-influenced lymphatic tissue to exogenous thyrotropin is dependent on a difference in the tendency to proliferation, localized directly to the lymphatic tissue. The involuted and still involuting lymphatic tissue would thus have a greater sensitivity of thyroid hormone (and perhaps to other stimuli as well), which can hasten regeneration. The raised concentration of thyroid hormone produced by the exogenous thyrotropin would be able, by this means, to bring about greater lymphatic growth than in normal animals without involution.

It is conceivable that a similar mechanism might be responsible for the fact that irradiation producing thymo-lymphatic involution has been found, both clinically and experimentally, to lead to subsequent excess morbidity in leukaemia or thymo-lymphatic neoplasm.

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Relative Erythrocyte Content in the Thyroid Gland as Measurement of the Gland's Activity

By

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Abstract

GYLLENSTEN, L., B. JALLING and U. TIDÉN. Relative Erythrocyte Content in the Thyroid Gland as Measurement of the Gland's Activity. *Acta physiol. scand.* 1959. *47.* 328—332. — In young guinea pigs, determinations were made of thyroid weight, mean acinar cell height and relative erythrocyte content per unit of thyroid section area. Thyrotropic hormone in doses of 1/8 to 4 guinea pig units daily for three days increased the blood corpuscle content, while thyroxine in single doses of 2—32 μg decreased it. The functional variations of the weight of the thyroid gland revealed greater correlation with the variations of the relative number of blood corpuscles than with the cell height variations, signifying that the weight variations partly depend on changes of blood content.

Colouring red blood corpuscles in histologic sections and counting them per standard mass of tissue constitutes a criterium of the relative quantity of blood vessels, mainly capillaries, and their functional use. Observations of the blood filling in capillaries have been made, inter alia, by KROGH (1929) when investigating physiologic and other variations in the functional conditions of the muscular tissue. The method has been worked out quantitatively by SJÖSTRAND (1934, 1935) and employed by him in investigations of the variations of blood content in diverse organs under certain different physiologic and pathologic conditions.

Under the assumption that all the capillaries in the tissue to be studied are injected with red blood corpuscles, the method also allows of quantitative determinations of the anatomic mass of capillaries. Such estimations in reference to the brain have been carried out, among others, by PETRÉN (1938) and LINDGREN (1940) and have shown correlations between the respective differentiation of the capillarization degree and the physiologic activity. In these investigations PETRÉN killed his animals with histamine injections, upon which the capillaries dilated and were filled with blood. Without such treatment counting of the blood corpuscles in a histologic section affords no information as to the entire anatomic quantity of capillaries in the tissue, but only of the part functioning at the time of death. Investigations of such physiologic capillarization in different organs, including some endocrine glands, and of the daily rhythmic variations of physiologic capillarization have been published by ENGSTRÖM, HOLMGREN and WOHLFART (1938). These authors also studied the thyroid gland of rats and mice but found no regular daily rhythmic changes of blood content in this organ. The individual variations of blood corpuscle content in the thyroid were very great.

The aim of the present investigation was to study whether there is any connection between the relative quantity of erythrocytes in sections of the thyroid gland and the functional condition of the gland. Thyroid activity was varied experimentally by means of injections of thyrotropic hormone, which stimulates the activity, and thyroxin, which inhibits it.

Technique

Male guinea-pigs of 200—250 g body weight were employed. Prior to the tests the animals were placed in their pens 1—2 weeks for adaptation. Those series for injection and the controls were prepared during the same period. The animals were killed at the same time of day.

Of the animals 12 were controls and 25 injected s.c. with thyroxine (Roche) and were killed 3 days later. These animals were divided into 5 groups of 5 and were given 2, 4, 8, 16 and 32 μg thyroxine per 100 g body weight, in a dilution of hormone 1 mg per 50 or 25 ml solution. 36 animals were treated with thyrotropic hormone (Ambinon, Pharmacia) i.p. during 3 days in succession, with preparation 24 hours after the last injection. Those treated with thyrotropin were divided into 6 groups, the animals of which received daily 1/8, 1/4, 1/2, 1, 2 and 4 guinea pig units of thyrotropic hormone, respectively. The dilution amounted to 0.2—1.0 ml per animal and injection.

The animals were killed with 3 ml 20 % urethane i.p. After the heart had ceased to beat, the thyroid gland was immediately dissected and weighed fresh, after which one lobe was fixed in 10 % formaldehyde for cell height measurement, and the other in potassium bichromate-sublimate-formaldehyde according to HELLY (ROMEIS 1948 § 337) for staining of the blood corpuscles. According to measurements of cell height by BORELL (1945) there is good agreement between the two lobes regarding the degree of histologic activity. The time of fixation was 12 hours.

The preparations were imbedded in paraffin and cut in thin sections of 5 μ, of

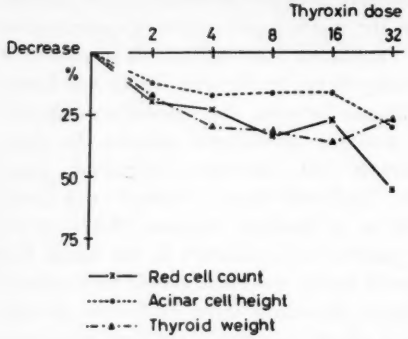

Fig. 1. Decrease of thyroid weight, acinar cell height and number of red blood corpuscles per standard area of section in the thyroid gland of guinea pigs, three days after the injection of 2—32 μg of thyroxine per 100 g body weight. The decrease is expressed in percentage of normal values. Each point represents the mean value of determinations of 5 animals, with the exception of normal values based on 12.

which at least 10 were taken from the central parts of the glandular lobes. The preparations fixed in formaldehyde were stained with iron chloride-hematoxylin in accordance with HÄGGQVIST's regressive method (ROMEIS 1948 § 682). The Helly-fixed preparations were coloured with diluted, buffered Giemsa solution 12 hrs at pH 5, the acidophil erythrocytes appearing distinctly red, in good contrast with the background.

The measurements of cell-heights were made with the aid of an integration ocular (Leitz) and the application of immersion optics (objective 95 ×, ocular 10 ×), at which the displacement of the movable line in the ocular could be read with a precision of 0.06 μ. Twenty-five cells were measured on each prepared section, all of which were chosen at random according to GYLLENSTEN's description 1953. The measurements of the blood corpuscles were performed with the same optics, and the total number of corpuscles per range of sight was counted on 25 ranges per thyroid. Under these optical conditions each range comprised an area of 1.50 mm². Only the capillary blood corpuscles were estimated. Fields of vision with larger vessels were avoided.

Results

The mean value of thyroid weight of normal animals was 46.2 mg (mean body weight 212 g), of acinous cell height 5.12 microns and of the number of blood corpuscles 28.8 per field of view.

Animals treated with thyroxine showed a decrease of thyroid weight, acinar cell height and relative amount of blood corpuscles (Fig. 1). Estimated on all the treated groups, accordingly independent of the dose, these decreases were statistically highly significant (p less than 0.001). Decrease of the relative quantity of blood corpuscles revealed greater correlation with the weight decrease (correlation coefficient 0.69 ± 0.13) than did the decrease of mean cell height (correlation coefficient 0.23 ± 0.18).

The thyroid glands of the guinea pigs treated with thyrotropin had an increase of weight, mean acinar cell height and relative number of blood corpuscles (Fig. 2). The calculation of all the treated groups, apart from the dose, reveals that the increases thus mentioned are statistically highly

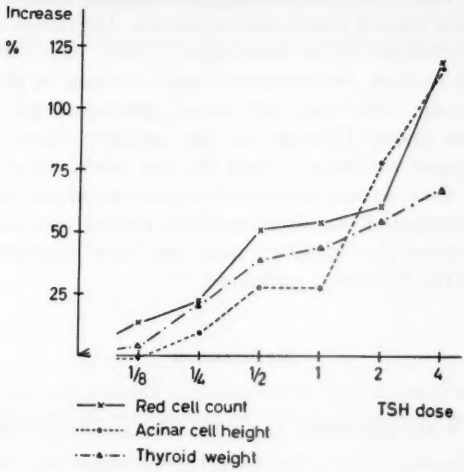

Fig. 2. Increase of thyroid weight, acinar cell height and number of red blood corpuscles per standard area of section in the thyroid gland of guinea pigs after treatment with 1/8—4 guinea pig units of thyrotropic hormone daily for 3 days. The increase is expressed in percentage of the normal values. Each point, with the exception of zero, represents 6 animals, zero 12 controls.

significant. Moreover, the thyrotropin treated animals displayed more distinct correlation between increase in content of filled blood vessels and weight increase than between the enhancement of cell height and weight increase (correlation coefficients 0.71 ± 0.12 and 0.32 ± 0.19).

Discussion

The results indicate a good correlation between the functional condition of the thyroid gland and the relative blood corpuscle content in the gland, determined through sectional calculation of blood corpuscles. The content of blood corpuscles varies as do the other activity criteria (acinar cell height and thyroid weight) in an approximatively logarithmic relation to the dose of the stimulating thyrotropin. A similar relation was found between the activity criteria and the dose of the administered thyroxine, which depressed the endogenous production of thyrotropin. The relatively good correlation between changes in blood corpuscular content and gland weight obviously indicates that part of the gland's weight variations under different activities, can be ascribed to variations of blood amount.

The present changes, found in the red cell content of the thyroid, when

activity changes took place, correspond to changes, known since long, in thyroid blood flow during functional variations. Literature on this subject has been recently referred to by SÖDERBERG (1958) who, with the aid of a delicately revised method, demonstrated quick changes in the flow of blood through the thyroid with even very small pharmacologic and physiologic influences on the gland. Changes in the capillary blood content of the thyroid in functional variations, found in our work, show that such increases of blood flow, in any case under certain conditions, are accompanied by an increased demand of the whole capillary volume and that this increased demand of the glandular capillary mass can vary quantitatively in good correlation with the functional variations.

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On the Development of Cortical Activity in Fetal Sheep

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Abstract

BERNHARD, C. G., I. H. KAISER and G. M. KOLMODIN. On the development of cortical activity in fetal sheep. *Acta physiol. scand.* 1959. *47.* 333—349. — An electrophysiological analysis was made of the prenatal development of the cortical activity in 17 non-anaesthetized sheep fetuses (calculated gestational age 65—151 days) kept in placentar contact with the decerebrate ewe. The pattern of prenatal development of the spontaneous and evoked cortical activity recorded from the exposed fetal cortex is described. An early prenatal activity (PN I activity) consisting in waxing and waning, temporary, regional, spindle-like bursts of regular waves was found as the only sign of cortical activity before the 78th day. During this prenatal period there was no sign of afferent cortical projection. Around the 80th day an irregular activity (PN II activity) added to the picture and the compound activity became continuous and extended over the cortex. At the same time cortical responses to afferent stimulation appeared, indicating the existence of thalamo-cortical functions. During the last part of the prenatal period characterized by a dominating PN II activity cortical arousal reactions could be evoked indicating the appearance of reticular functions. Simultaneously the barbiturate effect on the cortical activity became similar to that in adults.

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A review of the current literature reveals that there are surprisingly few investigations in which electrical signs of neuron activity have been used for the analysis of the functional development of the central nervous system during the prenatal period.

The studies of cortical electrical activity during the prenatal period which have been made previously all center around the question of the time of appearance of spontaneous cortical activity. According to an early study of KORNMÜLLER (1935) rabbits do not exhibit any such activity until some days after birth and the same result has been reported by SJÖSTRAND (see BARRON 1941). In guinea pigs JASPER *et al.* (1937) found the first sign of cortical activity to appear between the 48th and 56th day of gestation, the total gestation period of guinea pigs being 65 days. FLEXNER *et al.* (1950) found a prompt appearance of the spontaneous as well as of the strychnine-induced cortical activity in the same animal after 46 days of gestation; no such activity was found on the 44th day. The appearance of the spontaneous cortical activity in guinea pigs after 46 days of gestation is of considerable interest since, at the same time, there are obvious microscopic changes in the developing nerve cells accompanied by an increase of certain enzyme activities (see FLEXNER 1952). The main point in these investigations was to estimate the time for the appearance of spontaneous cortical activity and in the early work by JASPER *et al.* no ontogenetic trend was found in the characteristics of the developing cortical activity. BARCROFT and BARRON (1939, cf. BARRON 1941), in their review of the development of mammalian neuromuscular mechanisms, argue that while in some mammalians (*e. g.* the rat; gestation period 21 days) the functions of the suprasegmental centers seem to appear almost simultaneously, they appear successively in the sheep (gestation period 150 days) as judged by studies of spontaneous behaviour and reflex movements in sheep fetuses in which transections at different suprasegmental levels were made (BARCROFT and BARRON 1937). Conclusions based on removal of suprasegmental influences on spinal reflexes also indicate that in the sheep the functional maturation of the cortex is slow. The present paper reports our exploratory electrophysiological investigations of the prenatal development of the cortical electrical activity in sheep.

Methods

The experiments were performed on 17 fetuses of 14 decerebrate sheep (weight 20—47 kg). The ewes employed were, with one exception (no. 11), of the ordinary Swedish variety which resembles closely in appearance and adult size the Welsh breed employed extensively in Great Britain for experimental purposes. Since the ewes were settled in the field, exact durations of pregnancy are not known. Estimated durations have therefore been calculated from the equations of HUGGETT and WIDDAS (1951) for Welsh lambs using the separate values for singles and twins where appropriate. There are obvious unavoidable discrepancies where individual weights in a pair of twins differed greatly. The exceptional ewe, no. 11, was of a breed which is closer to the American

grade Rambouillet in its proportions. The estimated gestational age for its fetus was therefore calculated from an unpublished equation of Kaiser and Cummings for the Rambouillet breed, $W^{\frac{1}{3}} = 0.168\ (t - 41.4)$ for single lambs.[1]

The ewe was decerebrated after bilateral carotid ligation and tracheotomy. This was performed under the influence of thiogenal (methylthioethyl-2[1]-pentylthiobarbituric acid, sodium salt), a barbiturate which is rapidly destroyed and eliminated. On an average 35 mg of thiogenal per kg (23—49 mg/kg) were injected intravenously. About one hour after decerebration of the ewe the fetus was delivered by caesarean section through an incision between cotyledons and between umbilical vessels in the chorion. The fetus was then mounted on a small table which was fixed near the abdomen of the ewe, and care was taken not to disturb the umbilical circulation.

A polyethylene catheter was passed around the cord without tension and its limbs were put through a glass tube. The cord could be occluded under vision by pulling the catheter up through the tube to tighten the noose. Since this procedure regularly produced local spasm of both umbilical artery and vein, cord clamping was done only after all other basic observations had been made, and further studies were only undertaken if the spasm relented and fetal heart rate returned to normal frequencies.

In all experiments the head of the fetus was fixed in a stand. After craniotomy the edges of the skin flaps on each side of the sagittal incision on the head were lifted up so that a pool of liquid paraffin could be formed over the exposed brain. The preparation was kept warm with the aid of a heating lamp and the temperature of the pool (38° C) was monitored with a thermocouple.

Craniotomy was usually performed only on one side. The bone resection was extended just lateral to the midline in order to avoid bleeding. It was similarly limited posteriorly by the suture which overlies the junction of the parietal and occipital areas, to avoid bleeding in this area. Laterally and anteriorly the bone resection was carried as far as possible. The dura was easily removed, thus exposing the fronto-parietal cortex. The platinum electrodes for monopolar recording were gently placed at the cortical surface, the indifferent electrode being placed on the skull. In some instances the sciatic nerve and its branches were dissected free and kept in a paraffin pool made up of the surrounding muscles and the skin. The cortical activity of the fetus and the ECG:s of the ewe and the fetus were recorded with a 6 channel Grass electroencephalograph. For stimulation Grass stimulators were used.

The experiment usually started 2 hours after the injection of thiogenal, *i. e.* when the drug was eliminated. All recordings were thus performed on non-anaesthetized preparations. Immobilization of the fetal preparation with d-tubocurarine was necessary especially in the later phases of the prenatal period; a dose was given which abolished the spontaneous movements. When drugs were given to the fetus (d-tubocurarine, thiogenal) they were injected into the veins at the cotyledons. Injections into the main umbilical vessels must be avoided because of the susceptibility of these vessels to mechanical stimulation. An enduring constriction of the umbilical vessels which easily appears as the result of trauma greatly impairs the nervous activity of the fetus. In some cases the abdominal aorta of the ewe was dissected through an incision in the left flank. A plastic tube was put as a loose ligature around the aorta which then could be occluded at convenience by handling the ligature through a glass tube in the operative wound.

Three of the ewes experienced immediate respiratory arrest following decerebration, unrelated to the dose of thiogenal. This was treated with artificial respiration with oxygen through the tracheostomy for periods of a few minutes until spontaneous respiration was reestablished. Two other animals exhibited shallow respiratory patterns before and after decerebration. Most of the remaining ewes experienced brief periods of

[1] W = weight in g; t = age in days.

Table I

No.	Weight g	Calculated age (days)	Type of cortical activity (see text)	Cortical reactions to afferent stimulation (for explanation see text)
17	62	65	PN I	—
4	200	78	PN I	—
6	205	78	PN I	
8	370	87	PN I + PN II	—
5	450	90	» »	— (b)
13	785	100	» »	ac +
11	1,750	113	» »	ac +; t + (b)
10	1,300	112	» »	ac +
3	1,200	110	» »	—
7	1,490	115	» »	ac +
9A	1,300	128	» »	ac + (b)
9B	1,600	134	» »	
12	2,100	125	/PN I/ + PN II	ac + (b)
16A	2,100	144	» »	AR
14A	1,350	129	» »	—
14B	2,200	145	» »	ac + (b)
15	4,700	151	» » AD	AR

respiratory irregularity without disturbance of cardiac rate or rhythm immediately after decerebration. In general the ewes closer to term tended to experience more respiratory difficulty presumably because of the bulk of the uterus. Although the animals were starved at least overnight prior to operation gastrostomy decompression of the rumen was of some aid in reducing these difficulties.

Results

I. Spontaneous cortical activity

The experimental series comprised fetuses with weights from 62 g to 4,700 g. All fetuses showed spontaneous cortical activity which was relatively poor at the early developmental period and increased with the advance of the gestation period. For a systematic treatment of the results the material is assembled in Table I on the basis of the degree of gross anatomical differentiation of the brains (compare insets in Fig. 1—4). As seen in Table I the succession thus obtained agrees fairly well with the calculated ages of the fetuses.

Fig. 1 A shows the cortical activity in a 78 days fetus. The characteristic features of the corticograms at this stage are spindle-shaped bursts with a regular frequency of 13—14 per sec which appear against a background of electrical silence. These bursts often show an alternating appearance at different cortical points as seen in Fig. 1 A and B (in B, a higher speed of the paper was used than in A). They may, however, appear simultaneously in different regions as shown by Fig. 1 C obtained with still higher speed. The upper rec-

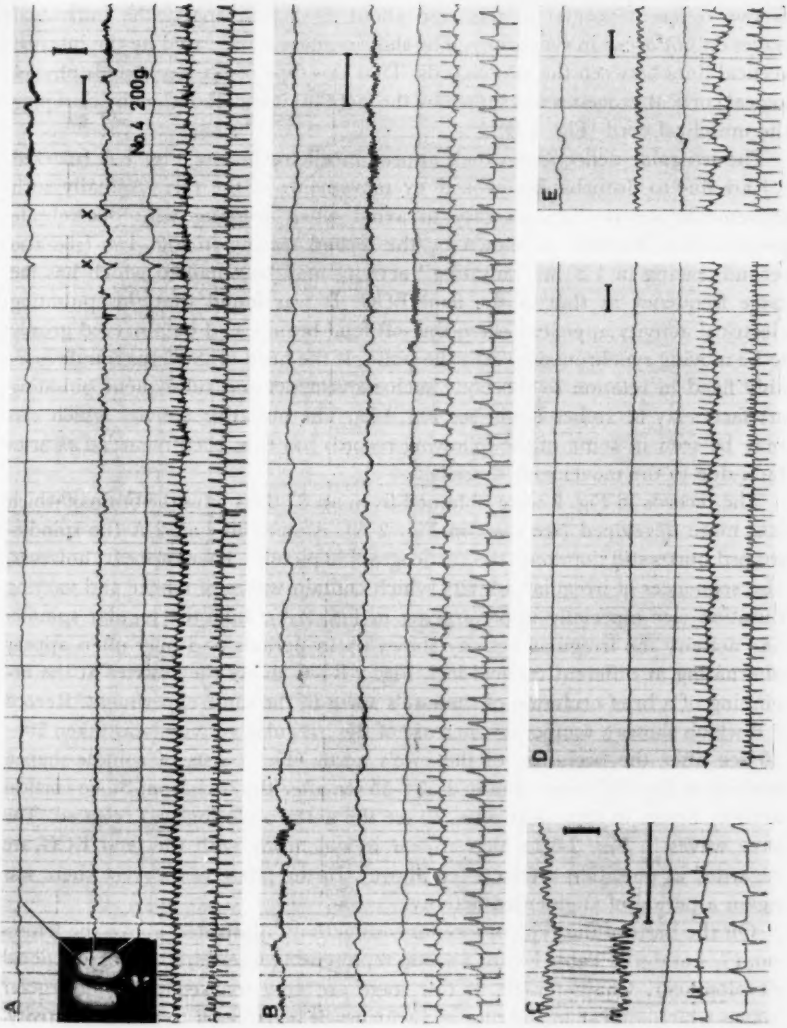

Fig. 1. Three upper tracings in A—E show spontaneous cortical activity from a 200 g fetus (calculated age 78 days). D, after clamping the umbilical cord. Thick horizontal line in A—E marks 1 sec. Thick vertical line in A marks 200 μV (same amplification in B, D and E) and in C 100 μV. The two lower tracings show the ECG of the fetus and the ewe respectively (same in all following figures). For further description see text.

ords in Fig. 1 C show that when such bursts of activity appear simultaneously at two different points (in this case about 2—3 mm apart) the individual waves do not occur in synchrony. The shift in phase is indicated by the inserted vertical lines between the two records. That this type of activity signals physiological cortical processes was shown by the fact that it was abolished by clamping the umbilical cord (Fig. 1 D).

The irregular deflections which appear in all tracings of Fig. 1 A (marked ×) are due to disturbances evoked by movements of the ewe. Actually such movements are not always easy to avoid when working with decerebrate preparations. Further, as shown by the second tracing in Fig. 1 A (see also second tracing in 1 B) a "pulsating" activity may be obtained which has the same frequency as that of the fetal ECG. It was found that this pulsating electrical activity appeared when the soft fetal brain could be observed grossly to be moving synchronously with the pulse. If the head of the fetus is unfavourably fixed in relation to the body such movements and subsequent pulsating artefacts may be rather large (see Fig. 1 E). The pulsating activity which also may be seen in some of the following records has thus been regarded as artefacts due to the movements described.

The records in Fig. 2 were obtained from an 87 days fetus the brain of which was more developed (see inset in Fig. 2 A). As seen in Fig. 2 A the spindle-shaped bursts still dominate the corticographic picture. There appear, however, also sequences of irregular activity which contain waves of longer and varying duration (see especially upper tracing in Fig. 2 A). Like the regular spindle-like activity the irregular activity shows up in periods and may often appear alternating at different cortical loci. Fig. 2 B—C shows the activity at the beginning of a brief occlusion of the ewe's aorta in the same experiment. Record 2 B which shows a temporary increase of the irregular activity, was taken 20—30 sec after the occlusion of the ewe's aorta. The bursts of spindle-shaped activity in Fig. 2 C were obtained 50—55 sec after the occlusion. Since cortical activity began to disappear after 70 sec the aorta occlusion was released. The slow waves in Fig. 2 C, which appear in synchrony with the fetal ECG, are regarded as pulsation artefacts (cf. above). In the phase of recovery there was again a period of augmented activity.

On the basis of the type of spontaneous activity illustrated above the fetuses no. 17, 4 and 6 in Table I form a group representing a certain stage of functional development. Characteristic of this stage are very marked trains of regular waves which appear in spindles and which will be referred to as PN I activity. The frequency of the individual waves which in Fig. 1 and 2 is about 13—14 per sec may vary between 12 and 18 per sec.

Later on (fetuses 8 and 5) periods of irregular activity also appear (PN II activity). Both these types of activity often appear temporarily against a silent background and especially in the early phase the active regions are circumscribed.

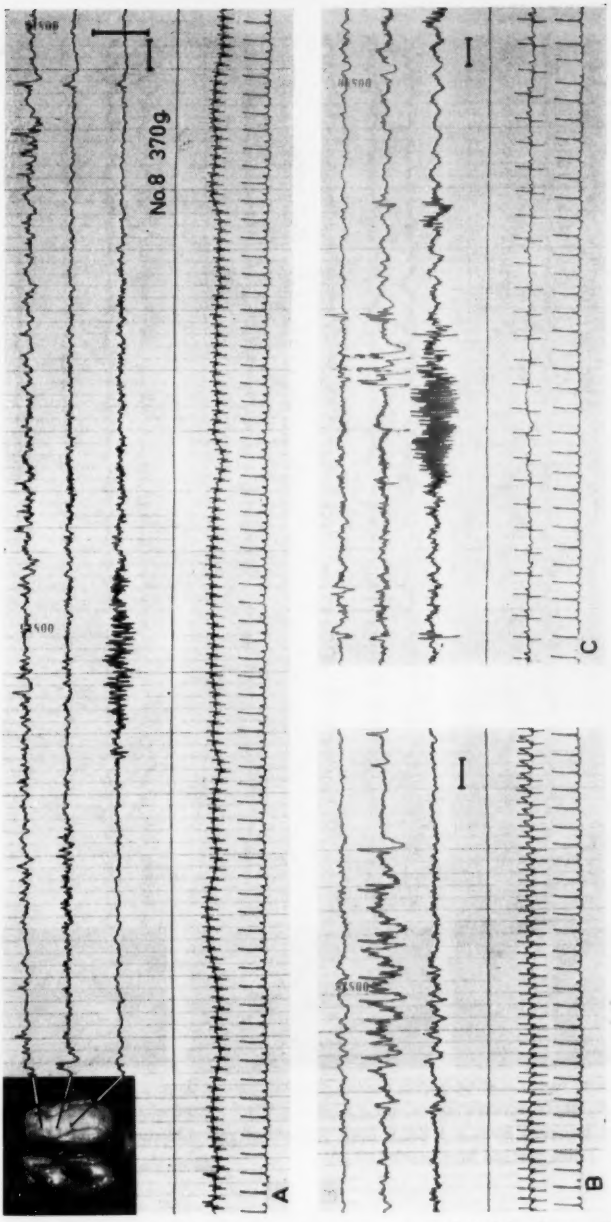

Fig. 2. Three upper tracings in A—C show spontaneous cortical activity from a 370 g fetus (calculated age 87 days). B, 20—30 sec and C 50—55 sec after occlusion of ewe's aorta. Thick horizontal line marks 1 sec. Thick vertical line in A marks 200 μV (same amplification in B and C).

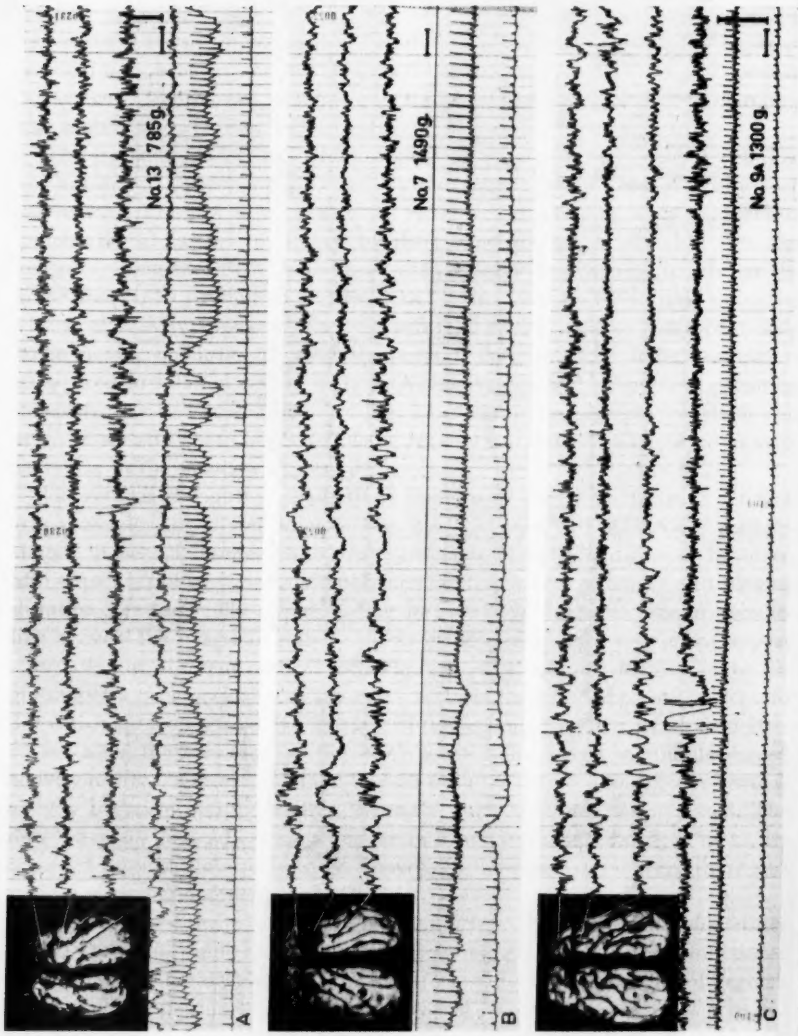

Fig. 3. A, (four upper tracings) spontaneous cortical activity from a 785 g fetus (calculated age 100 days). B, (three upper tracings) same from a 1,490 g fetus (calculated age 115 days) and C (four upper tracings) from a 1,300 g fetus (calculated age 128 days). Thick horizontal lines mark 1 sec. Thick vertical lines mark 200 μV.

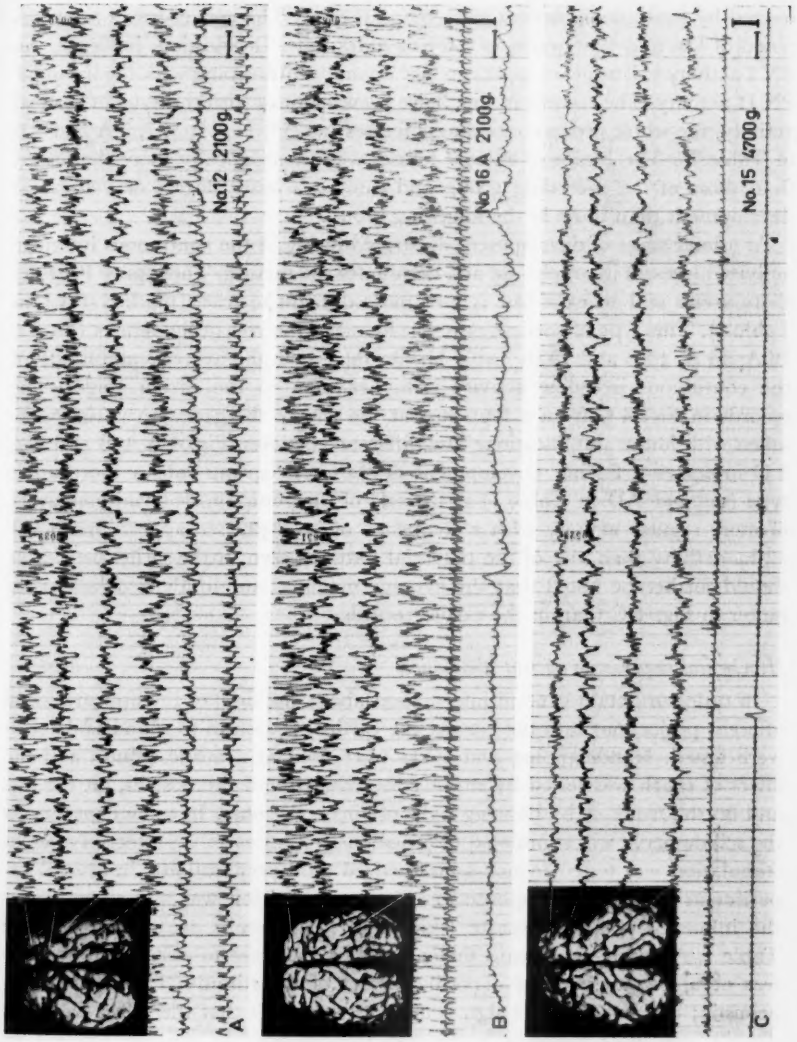

Fig. 4. Four upper tracings in A—C show spontaneous cortical activity in a 2,100 g fetus (A; calculated age 125 days), a 2,100 g twin fetus (B; calculated age 144 days) and a 4,700 g fetus (C; calculated age 151 days). Thick horizontal lines mark 1 sec; thick vertical lines mark 200 μV in A—B and 500 μV in C.

As seen in Fig. 3 there is a transition into a following stage which is characterized by continuous cortical activity in all leads. The corticograms are composed of fast and slow waves of varying amplitude. In addition, however, the PN I activity still appears superimposed upon, or intervening with the irregular PN II activity. The records in Fig. 3 are from 3 fetuses with this type of cortical activity, the whole group containing 7 fetuses (no. 13, 11, 10, 3, 7, 9 A and 9 B in Table I). The brains of these 7 fetuses were anatomically more developed than those of the preceding group and showed a lesser degree of anatomical development than those in the following group.

At a later stage of development illustrated in Fig. 4 the continuous irregular activity increases in amplitude and dominates the picture. The typical PN I activity seems still to exist but is now more difficult to trace (marked /PN I/ in Table I). This type of corticogram was found in the rest of the fetuses (no. 12, 16 A, 14 A, 14 B and 15 in Table I). On the whole the average amplitude of the continuous irregular activity is higher than in the earlier stages. The records in Fig. 4 C from a fetus just at the end of the gestation period were taken with lower amplification. The difference between Fig. 4 A + B and Fig. 4 C indicates a further development of the corticograms into a more adult type (marked AD in Table I) at the end of the gestation. Intermittent trains of more regular activity with a frequency of 7—8 per sec appear, more like alpha-activity than any of the potential patterns seen in the earlier periods. It should further be noted that the average maximal amplitude is considerably larger in Fig. 4 C than in the earlier records.

II. Cortical responses to afferent stimulation

In order to obtain general information about the time of the appearance of afferent projections into the cortex, the cortical responses to sound and touch were tested. Handclapping and clicks were used as acoustic stimuli and the effect of touch was tested by striking the skin around the nostrils, on the legs and on the trunk or by blowing puffs of air on the nose. In some experiments the sciatic nerve was stimulated electrically. In most cases the effect of afferent stimulation was tested in non-anaesthetized fetal preparations. In several experiments the cortical response to afferent stimulation was also tested under the influence of a barbiturate (thiogenal). The results are put together in Table I. A minus sign means that no cortical response to afferent stimulation was obtained, a plus sign a positive effect of the stimulus indicated (ac = acoustic; t = touch). The sign b in brackets means that the effect was most clearly seen after giving a barbiturate (thiogenal). As seen in Table I no cortical response to afferent stimulation was obtained at the stage characterized by the intermittent spontaneous cortical activity illustrated in Fig. 1 and 2 (no. 4, 8 and 5 in Table I; no. 6 was not tested). When the functional development of the brain reached a stage characterized by a widespread continuous spontaneous activity (confer Fig. 3 A), cortical responses to afferent

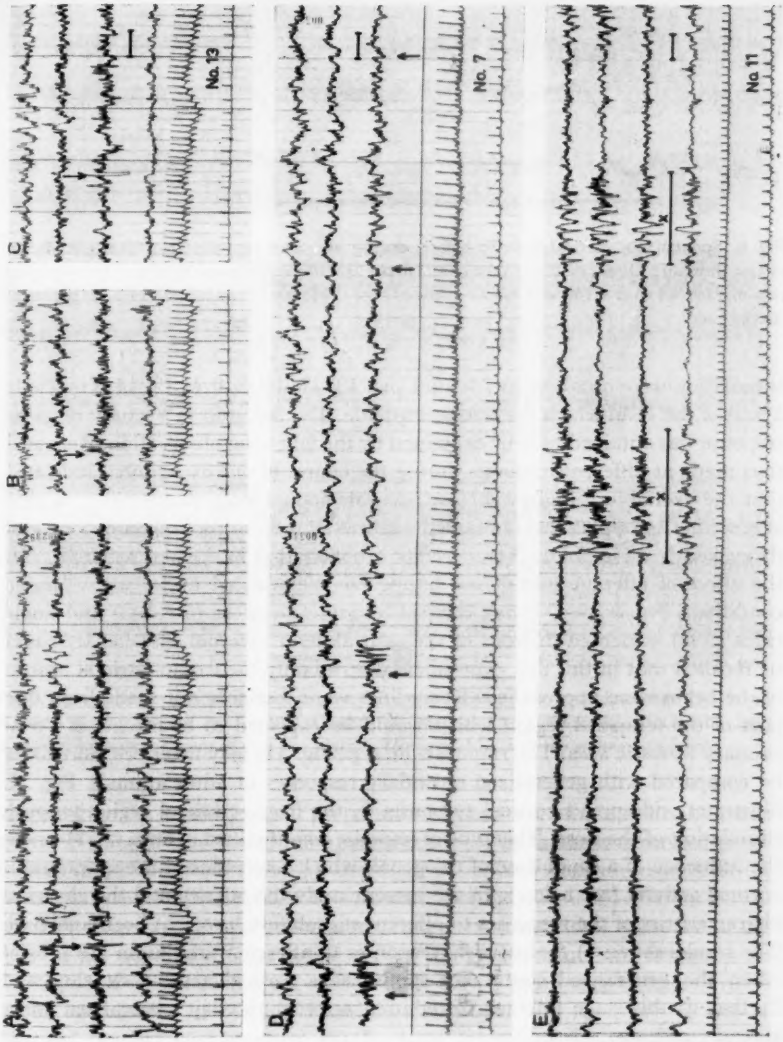

Fig. 5. A—C, (four upper tracings) spontaneous cortical activity and cortical responses (at arrows) to single clicks in a 785 g fetus (calculated age 100 days). D, (three upper tracings) spontaneous cortical activity and cortical responses to single claps (at arrows) in a 1,490 g fetus (calculated age 115 days); E, (four upper tracings) spontaneous cortical activity and cortical response to tactile stimulation of the nose (at ×) in a 1,750 g fetus (calculated age 113 days). Horizontal lines to the right in the records mark 1 sec.

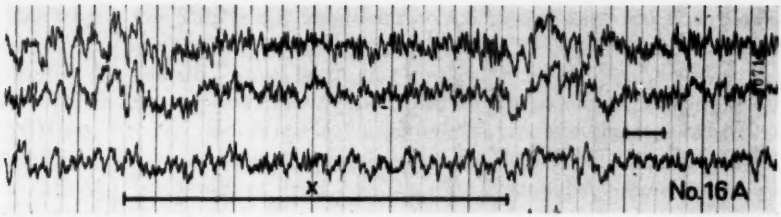

Fig. 6. Spontaneous cortical activity before, during and after repetitive electrical stimulation of low threshold fibres (see text) in the sciatic nerve (duration of stimulus indicated by horizontal line marked ×) in a 2,100 g fetus (calculated age 144 days). Horizontal line to the right marks 1 sec.

stimulation were obtained in 7 fetuses (no. 13, 11, 10, 7, 9 A, 12, 14 B in Table I) out of the 11 in which the tests were made. The fact that in 4 fetuses no clear response was obtained may be explained by the following observation. Repeated tests made at different instances during the course of one experiment indicated that the excitability underwent considerable spontaneous fluctuations, so that at one time no response was obtained whereas at another the responses were easily evoked (see Fig. 5 D). It is therefore probable that in the four negative cases the effect of afferent stimulation happened to be tested under unfavourable conditions. Fig. 5 A—D shows cortical responses to clicks (5 A—C) and single claps (5 D) presented in front of the contralateral external meatus. It should be recalled that in this first experimental series only the fronto-parietal aspects of the brain were approached. Recordings were therefore not made from that part of the temporal region which would be expected to be the site of a fetal primary acoustic area. The responses in, *e. g.*, Fig. 5 D may therefore more likely be compared with generalized secondary responses in adult animals. Fig. 5 E illustrates widespread cortical responses in the fronto-parietal region to touch stimulation of the nose. This type of response was obtained in fetus no. 11 under the influence of a small dose of thiogenal, which had reduced the spontaneous cortical activity (see below). In the present study the latency and the electrical characteristics of the responses to afferent stimulation have not been analyzed. The results show: 1) that cortical responses to afferent stimulation are present when the generalized continuous spontaneous cortical activity develops and 2) that at this stage afferent stimulation appears to elicit generalized widespread responses.

In a later phase it was also possible to obtain a certain type of "cortical arousal reaction". Such a reaction is illustrated in Fig. 6 from fetus 16 A (AR in Table I) which shows how the irregular high-amplitude cortical activity changes into a high-frequency, low-amplitude activity during electrical sciatic stimulation. Actually the strength of the sciatic stimulation was just above threshold for the elicitation of a monosynaptic spinal cord reflex response which

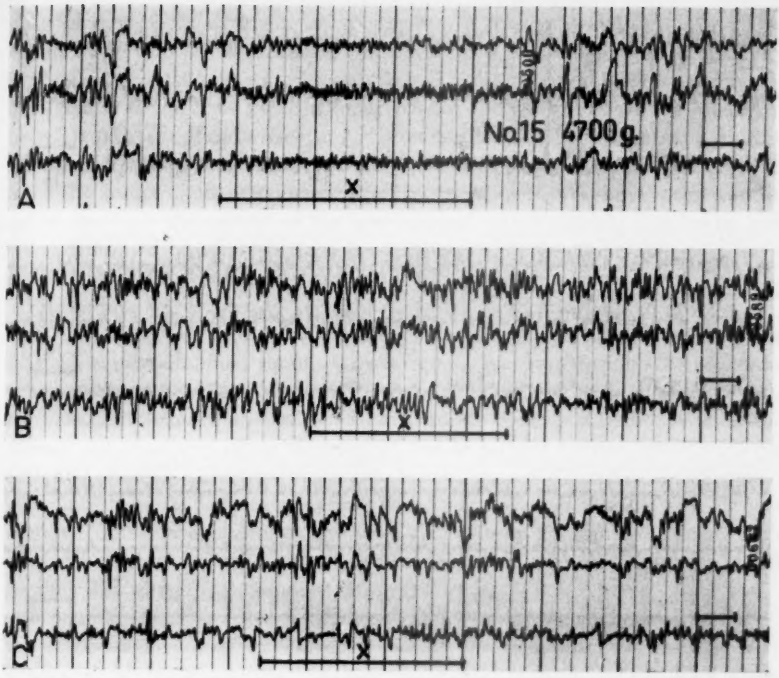

Fig. 7. Spontaneous cortical activity from a full term 4,700 g fetus (calculated age 151 days). A before, B after 10 mg per kg and C after 40 mg per kg of thiogenal. Horizontal lines marked × indicate duration of skin stimulation. Horizontal lines to the right mark 1 sec.

happened to be tested in this preparation. When the stimulus strength was increased general avoidance movements occurred in this non-curarized preparation, which indicated that high-threshold pain fibers were stimulated. Thus, the arousal reaction was evoked by stimulation of low-threshold afferent fibers. Fig. 7 A shows an arousal reaction to cutaneous stimulation (blowing of air puffs on the nose) of a fetus at the end of the gestation period. It should be noted that the cortical arousal responses in Fig. 6 and 7 A are characterized by the appearance of the PN I activity (cf. Fig. 1 and 2). After 10 mg of thiogenal per kg were given to the fetus the arousal reaction disappeared as seen in Fig. 7 B.

In this context should also be mentioned our observations on *the effect of a barbiturate* on the spontaneous cortical activity in the fetuses. As mentioned above thiogenal was used because of its rapid elimination. Injection was given slowly into a cotyledonary vessel until a change in the spontaneous activity was obtained. In fetuses belonging to the earlier stages (no. 4—9 B in Table

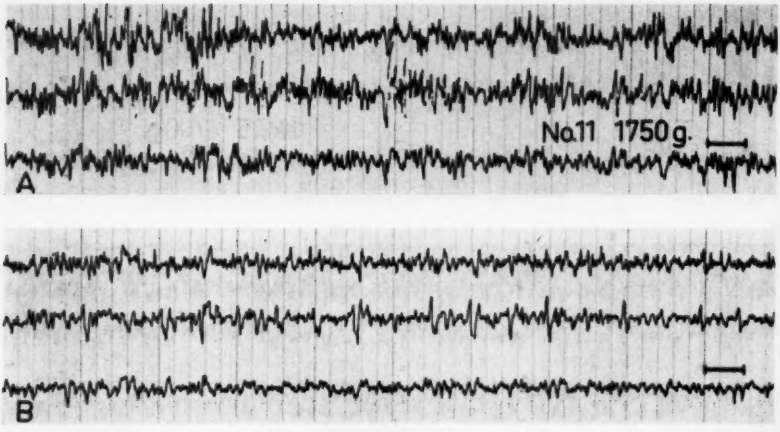

Fig. 8. Spontaneous cortical activity from a 1,750 g fetus (calculated age 113 days). A before, B after 10 mg per kg and C after 40 mg per kg of thiogenal. Horizontal lines mark 1 sec.

I) only a reduction of the spontaneous activity was seen (see Fig. 8 A—C). Only in the late phase of the PN II stage did an increase of the continuous irregular activity appear (Fig. 7 B) at doses lower than those which ultimately resulted in a reduction of the cortical activity (Fig. 7 C). The amount of thiogenal which in fetus no. 15 produced this barbiturate activity (Fig. 7 B) also caused abolition of the arousal reaction as described above. It should be noted that the doses are calculated without correction for the weight of the placenta.

Discussion

As has been pointed out by BARCROFT and BARRON (1939) there are several advantages in using the sheep for studies in functional neuroembryology. During the long gestation period (150 days) the central nervous system reaches a comparatively high degree of maturation as judged by the behaviour of the newborn lamb. Thus, the sheep offers good opportunities for a differentiation of successive developmental stages and the nervous functions can be followed on the same type of preparation, from the very early stages up to a

stage (*i. e.* at birth) when the nervous system is relatively well developed. Our studies show that the mechanisms underlying the spontaneous cortical activity as well as the processes for afferent cortical projection reach a high degree of maturation in the fetal sheep compared with the cat as judged by investigations on newborn kittens (see *e. g.* GROSSMAN 1955). These postnatal studies were performed on barbiturate preparations and in some cases by leading off from the intact skull of non-anaesthetized newborns. We have been careful not to base our description of the development of the cortical activity on any results obtained on anaesthetized preparations since we found that even very small doses given through the umbilical vein abolish the cortical activity.

Precautions were taken to keep the umbilical cord and fetus in good condition (see Methods). However, we are aware of the necessity of pointing out that the results obtained should be referred to the preparation used since, as has been stressed by BARCROFT and BARRON (1939) as well as by WINDLE (1944), the changes in environmental conditions due to the exposure of the fetus influence the reactivity of the nervous system as judged by movement patterns and these changes increase with the time after exposure. Therefore the technical procedure from delivery to recording was made as fast as possible and we also tried to limit the exposure of the deep tissues to a minimum. Under such conditions the functions tested showed a remarkable constancy which lasted considerably longer in the more mature fetuses.

Even the youngest fetus of this exploratory series which had a calculated age of 65 days (no. 17 in Table I) showed the temporary regional activity characterized by regular waves in waxing and waning spindle-like bursts which has been called PN I activity. As judged by the observations on the three youngest fetuses in the material this type of activity seems to represent the earliest prenatal sign of spontaneous cortical activity. Further investigations on still younger fetuses are being carried out in order to find out the earliest date for its appearance as well as its relation to the embryonic cortical structures. Obviously this activity appears in circumscribed regions of varying extension. The PN I type of activity appears alone during a certain fetal period before the appearance of the irregular activity (PN II activity) which later on adds to the picture. Successively the cortical activity comprising both the PN I and PN II fractions becomes continuous and expands over the cortex. It should also be mentioned that post-stimulatory after-discharge in response to cortical stimulation was not obtained until this stage of development was reached (see BERNHARD, KAISER and KOLMODIN 1959). It may well be that the transition into an integrated continuous form of activity (PN I + PN II in Table I) depends on the development of subcortical functional connections since in the material so far tested the evoked cortical responses appeared at this stage of development (see Table I), indicating the appearance of thalamocortical functions. It is well known from classical investigations that at this stage widespread reflex movements in the neck and limbs can be evoked by

stimulation of the skin in the facial region and on the limbs (see *e. g.* BARCROFT and BARRON 1939). We have also been able to record the electrical signs of reflex activity from the spinal cord and nerves. Thus, peripheral afferent systems and certain spinal and bulbar centers activated by tactile stimulation transmit impulses at this stage of development. It may therefore be suggested that the PN I fraction represents an inherent cortical activity which may appear independently of a subcortical drive. The PN I type of activity reminds of the temporary potential bursts from the cortex which according to BREMER (1949) may appear in the cortex of adult animals (cat) after deafferentation by undercutting. On the other hand it has been stressed that an isolated cortical gyrus does not show any spontaneous cortical activity under physiological conditions (BURNS 1951). In this context it should, however, be pointed out that the appearance and maintenance of the embryonic PN I activity may be facilitated or even initiated by alterations in the O_2 and CO_2 content of the fetal blood induced by the manipulation of the uterus and fetus. According to WINDLE (1944) opening of the uterus is followed by fetal hypoxia which considerably alters the reactivity of the central nervous system. As seen in Fig. 1 an occlusion of the umbilical cord may enhance the PN I activity before the block appears.

The PN I activity seems to remain throughout the whole fetal life but during the last prenatal period when the corticogram changes into an adult type the PN II activity dominates the picture to such a degree that the PN I activity becomes difficult to trace (indicated by /PN I/ + PN II in Table I). At this stage a "cortical arousal" to different types of afferent stimulation can be evoked which would indicate the maturation of reticular and intralaminar projection functions. At the same time barbiturate effects comparable to those in adult animals are obtained. It should be noted that during the period of blocking of the high-amplitude irregular activity, characteristic of the arousal reaction, the PN I type of activity seems to appear unmasked.

Our results show a very definite trend in the ontogenetic development of the cortical activity. It is also obvious that in the sheep the successive developmental stages are spread out over a long period during the prenatal life. We still do not know the exact date for the first appearance of the PN I activity which so far has been found in a 65 days fetus. According to neurohistological studies which are being carried out (ÅSTRÖM, personal communications) there is a beginning lamination of the cortical cells at this stage. If the above conclusion is right, viz. that the PN I fraction represents an inherent activity of the embryonic cortical cells which may appear independently of a subcortical drive it may be that this type of activity exists at a very early developmental stage.

On the basis of experiments on guinea pig fetuses which become relatively mature after a short gestation period (65 days) it has been claimed that the cortical electrical activity appears abruptly between the 44th and 46th day

of gestation (FLEXNER *et al.* 1950). The cortical activity referred to, which according to FLEXNER (1952) shows an interesting correlation to important changes in the enzymatic systems of the brain, seems to represent the type which we have described as PN II activity. In our sheep material so far tested this develops between the 80th and 100th day of the 150 days long gestation. The embryonic PN I type of activity characterized by intermittent appearance may have been overlooked in the experiments on the fairly small brains of the guinea pig fetuses delivered before the 44th day. It is interesting to note that in recordings from the brain of a 43—44 days old human fetus (BORKOWSKI and BERNSTINE 1955) an activity has been found which reminds of the PN I activity. This activity from the human brain was recorded immediately after delivery by caesarean section.

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Occurrence and Distribution of Catechol Amines in Brain[1]

By

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Abstract

BERTLER, Å. and E. ROSENGREN. Occurrence and distribution of catechol amines in brain. *Acta physiol. scand.* 1959. *47*. 350—361. — The occurrence and distribution of noradrenaline and dopamine in mammalian brain have been investigated. In all the mammalian brains investigated noradrenaline and dopamine are present in about the same amounts. Noradrenaline is localized mainly to the brain stem. The distribution of dopamine differs greatly from that of noradrenaline, the highest concentrations being found in corpus striatum and only small amounts in other brain parts. In the brain parts which are rich in catechol amines, the activity of DOPA decarboxylase is high, too. Dopamine is suggested to play a role in the control of motor functions.

The occurrence in brain of "sympathin", with properties resembling noradrenaline rather than adrenaline, was demonstrated by EULER in 1946. HOLTZ (1950) presented evidence to show that the brain "sympathin" was composed of 90 per cent noradrenaline and 10 per cent adrenaline. VOGT (1954) showed that these amines were unevenly distributed in brain, the highest concentrations being observed in the brain stem, notably the hypothalamus. A third catechol compound, suggested to be dopamine, was demonstrated in brain by MONTAGU (1957). Independent work in the present laboratory provided definite evidence that dopamine is a normal brain constituent (CARLSSON *et al.* 1957). Data on the occurrence and distribution of

[1] A preliminary report of part of this work has been published earlier (BERTLER and ROSENGREN 1959).

Table I. Stability of Noradrenaline (NA) and Dopamine (DA) in the Rabbit's Brain after Extirpation

The figures stand for μg per g tissue

Hours after extirpation					
0		1		3	
NA	DA	NA	DA	NA	DA
0.36	0.38	0.30	0.20	0.18	0.24
0.44	0.38	0.43	0.33	0.18	0.27
0.35	0.27	0.27	0.21	0.18	0.20
		0.27	0.24		
Mean 0.38	0.34	0.32	0.25	0.18	0.24
Catechol amine content as per cent of the amount found in brains immediately extracted					
100	100	84	74	47	71

these catechol amines in brains of different species will be presented and discussed below.

It was also considered of interest to study the distribution of l(—)dihydroxyphenylalanine decarboxylase (DOPA decarboxylase), which is known to occur in brain (DIETRICH 1953, HOLTZ and WESTERMANN 1956) and generally believed to be responsible for the formation of dopamine from DOPA in the body. Dopamine can then be converted to noradrenaline by hydroxylation in the side chain, and is considered to be the normal precursor of noradrenaline (BLASCHKO 1957).

Experimental

The brains of the cows and pigs and two of the sheep were received from the slaughter house. The brains of the dogs were removed after the animals had been bled to death under nembutal anaesthesia. Cats were anaesthetized with N_2O and decapitated. Other animals used in this investigation (rabbits, guinea pigs, rats and two sheep) were killed by a blow on the neck and bled to death.

In order to study the stability of the brain catechol amines during these preparations, brains of normal rabbits were dissected out and put into stoppered beakers. They were kept at 21°—22° C for various time intervals before they were extracted with perchloric acid solution. The results are given in Table I. A lowering of the noradrenaline and dopamine content gradually occurred when brains were left at room temperature. After three hours about 50 per cent of the noradrenaline and 70 per cent of the dopamine were left (Table I). In other experiments rabbit brains were divided into the cerebral hemispheres, the brain stem and the cerebellum and kept at room temperature for various time intervals. The lowering of the catechol amine content was found to be about the same in the three brain parts.

In the experiments performed to investigate the catechol amine content in whole brains (Table II), the time between death of the animal and homogenization of the

Table II. Occurrence of Noradrenaline and Dopamine in Brains of Different Animals

	Number of animals	Noradrenaline µg/g	Dopamine µg/g
Rat	5	0.49	0.60
Guinea pig	3	0.38	0.34
Rabbit	9	0.29	0.32
Cat	6	0.22	0.28
Dog	2	0.16	0.19
Pig	2	0.14	0·22
Sheep	3	0.21	0.29

Table III. Distribution of Noradrenaline (NA) and Dopamine (DA) in Different Parts of Brain

The figures stand for µg per g tissue

Figures in brackets refer to number of animals

Part of the brain	DOG (2)		SHEEP (2)		PIG (2—3)		CAT (3)	
	NA	DA	NA	DA	NA	DA	NA	DA
Cerebral hemispheres (not corpus striatum and hippocampus)								
rostral part	0.13	0.07	0.16	0.12	0.09	0.08	0.22	0.08
caudal part	0.12	0.08	0.13	0.02	0.09	0.05	0.23	0.10
Caudate nucleus	0.10	5.90	0.13	6.68	0,21	6.1	0.22	8.00
Lentiform nucleus	0.08	1.63	0.10	4.78	0.03	3.6	0.20	1.90
Hippocampus	0.14	0.13	0.15	0.06	0.09	0.04	0.14	0.08
Hypothalamus	0.76	0.26	1.15	0.19	0.83	0.88	2.01	0.75
Diencephalon (not hypothalamus)	0.17	0.09	0.28	0.16	0.18	0.11	0.34	0.16
Mesencephalon	0.33	0.20	0.47	0.24	0.31	0.14	0.43	0.19
Pons	0.41	0.10	0.58	0.04	0.25	0.06	0.52	0.11
Medulla oblongata	0.37	0.13	0.46	0.17	0.22	0.05	0,39	0.08
Cerebellum	0.06	0.03	0.06	0.03	0.10	0.01	0.13	0.02

tissues in perchloric acid solution was generally not more than a few minutes. In experiments carried out to determine the distribution of the catechol amines in brain pieces, the meninges were stripped off from the brain tissue, and the brain divided into various parts (Table III). These preparations were carried out at low temperature and were not allowed to last more than 30 min.

It may be concluded from these data that the loss of catechol amines prior to the extraction was slight. As regards losses in the following steps recovery experiments run in the course of the investigation showed that about 90 per cent of noradrenaline and 85 per cent of dopamine added to the extracts were recovered.

Figure 1. Paper Chromatography of Catechol Amines in Extracts Containing Ferric Ions. A. 1st eluate of an extract containing 20 μg noradrenaline and 57 μg ferric chloride. The column had been washed with 10 ml redistilled water. B. The same as A, but the column had been washed with 10 ml of an EDTA solution. The chromatogram was run in butanol-HCl.

Determination of Catechol Amines

Extraction and purification were performed as described in a previous paper (BERTLER, CARLSSON and ROSENGREN 1958), except that a different type of Dowex 50 was used: X—4 instead of X—8. This change of resin required certain modifications of the procedure. In the present work each column contained 0.35 g (dry weight) Dowex 50, X—4, mesh size 200—400. Elution was performed by means of two portions (8 and 10 ml) of 1.2 N hydrochloric acid. Noradrenaline and adrenaline were almost quantitatively found in the first portion (1st eluate), dopamine in the second (2nd eluate).

The columns could be used for several determinations if they were washed with 20 ml 5 N hydrochloric acid between the runnings. They were buffered to pH 6 with 20 ml N sodium acetate-acetic acid buffer, passed through the column in small portions.

If the eluates were to be chromatographed on paper the interference of heavy metal ions had to be minimized by washing the columns with 25 mg of the disodium salt of ethylene diamine tetraacetic acid (EDTA) dissolved in 10 ml of water and neutralized to pH 6.5. In our experience traces of ferric ions in the extracts constitute the most common contamination, which would interfere with the chromatography. When the chromatograms run in butanol-HCl were sprayed with potassium ferricyanide solution (see below), the presence of a few micrograms of $FeCl_3$ in the eluates gave a distinct blue spot near the start-line and another weakly brown-coloured spot between the latter and the noradrenaline spot (Fig. 1).

Oxidation of catechol amines in the eluates was carried out as reported by BERTLER *et al.* (1958) and CARLSSON and WALDECK (1958). For the determination of dopamine, tissue blanks treated in the same way as the sample were used, except that the sodium sulfite solution was added immediately after the addition of acetic acid.

Specificity of the Methods

The specificity of the method for determination of noradrenaline and adrenaline has been investigated in an earlier paper (BERTLER *et al.* 1958). The specificity of the dopamine method has been further examined by means of quantitative paper chromatography. The procedure is essentially the same as that used in the earlier work on the specificity of the noradrenaline and adrenaline method. A detailed description

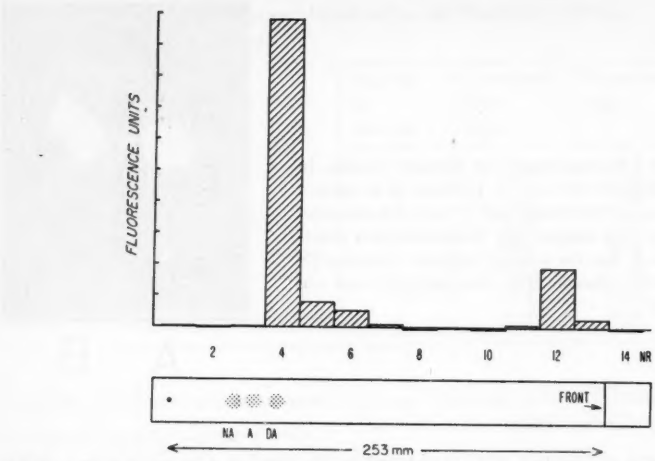

Figure 2. Quantitative Paper Chromatography of the 2nd Eluate from the Rabbit's Brain.
Activating wave-length 345 mμ.
Fluorescent wave-length 410 mμ.
For explanation see text.
Note. The activation and fluorescence spectra obtained from piece no. 4 were identical with those of dopamine. The amount of dopamine recovered from this piece was 3.56 μg corresponding to 0.29 μg per g tissue.

of our procedure for quantitative paper chromatography has not been given before and will therefore be presented below.

The dopamine content of the 2nd eluate from brains of rabbits was determined directly in an aliquot of the eluates. The remainder was evaporated to dryness *in vacuo*. The acid eluate was placed in a bottle, which was connected to a high vacuum pump *via* a chamber cooled by dry ice and a cylinder containing sodium hydroxide pellets. The latter served to trap any vapors escaping through the ice chamber. The bottle was immersed in a water bath at constant temperature (25° C). The evaporation was stopped immediately when the residue in the bottle looked dry, *i. e.*, when it had changed from grey to white. The residue was extracted twice with 2 ml acetone containing one per cent 0.1 N hydrochloric acid. The acetone extract was evaporated to a small volume and put on paper. A reference containing synthetic dopamine was run on the same paper. The paper (Whatman no. 1) had previously been washed with, in order, 0.5 N hydrochloric acid (6 to 8 hours), 96 per cent ethanol (24 hours) and water (8 to 10 hours). After ascending development for 16—24 hours at 26° C in n-butanol equilibrated with the same volume of 1.2 N hydrochloric acid, the paper was divided into two parts, one containing the reference and the other the brain materials. The former part was dried at 65° C and sprayed with a potassium ferricyanide solution (0.44 per cent potassium ferricyanide in 0.1 M phosphate buffer, pH 7.5). The latter part was dried in a vacuum chamber, which was connected to a vacuum pump *via* a cylinder surrounded by dry ice. The paper was then cut transversally into small strips. Each strip was eluted with 4 ml 0.01 N hydrochloric acid in an apparatus similar to that described by SJÖVALL (1955). The dopamine content of each eluate was determined. The results of such an experiment are shown in Fig. 2.

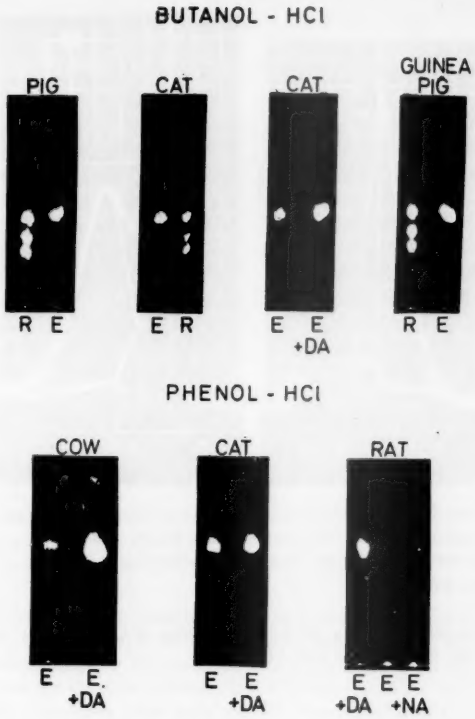

Figure 3. Paper Chromatography of the 2nd Eluates from Brains of Different Mammalian Species.

The upper chromatograms were run in butanol-HCl; the lower ones in phenol-HCl.

R: References (from bottom to top): noradrenaline, adrenaline and dopamine.

E: aliquot of 2nd eluate.

E + DA: 2nd eluate with added dopamine (5 or 10 μg) but otherwise identical to E on the same paper.

E + NA: 2nd eluate with added noradrenaline (5 μg) to the extract but otherwise identical to E on the same paper. As noradrenaline appears in the 1st eluate, it is not seen in this chromatogram.

Description of samples

Species	Tissue
Pig	Caudate nucleus, 0.7 g.
Cat (top left)	Caudate and lentiform nuclei, from one animal.
Cat (top right)	Whole brain, 13.5 g.
Cat (bottom)	Whole brain, 13.5 g.
Guinea pig	Whole brain, from four animals.
Cow	Whole brain, 9.3 g.
Rat	Whole brain, from two animals.

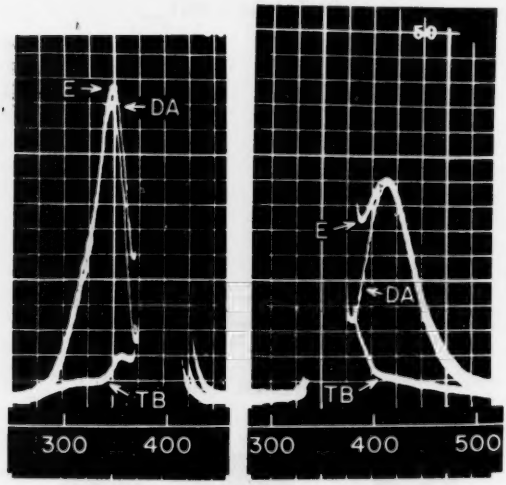

Figure 4. Activation and Fluorescence Spectra of 2nd Eluate from the Caudate Nucleus of the Cat.
To the left: Activation spectrum. Fluorescent wave-length 420 mμ.
To the right: Fluorescence spectrum. Activating wave-length 335 mμ.
E: 2nd eluate from brain.
TB: Tissue blank.
DA: Dopamine standard, 0.081 μg per ml. The spectra of DA were run at a sensitivity three times lower than the spectra of E and TB.

Of the dopamine originally present, 78 per cent were recovered from the strip corresponding to the dopamine reference. In three other experiments the corresponding figures were 70, 71 and 87 per cent. (Apparently a loss of 13 to 30 per cent had occurred during the evaporation and the following steps of the procedure.) The spectral characteristics of the material recovered from this strip were undistinguishable from those of authentic dopamine.

As seen in Fig. 2 some fluorescent material was recovered from the vicinity of the front. The spectral characteristics of this material differed from those of the dopamine fluorophore. The material does not seem to be derived from dopamine as it does not increase after addition of large amounts of dopamine. It was probably not included in the direct determination as it was found to persist in an extract from the brain of a reserpinized animal although the dopamine value of the original eluate was zero. The quantitative paper chromatography thus lends further support to the specificity of the dopamine method for brain.

The presence of noradrenaline and dopamine in the brains of the species listed in Table II was confirmed by paper chromatography of the eluates in butanol-HCl and phenol-HCl (BERTLER *et al.* 1958). The spots were visualized by spraying the paper with the ferricyanide solution mentioned above. Some chromatograms obtained from the 2nd eluate of whole brain or part of the brain are shown in Fig. 3. The 2nd eluates from whole brains or the caudate nuclei gave spots closely corresponding to authentic dopamine. The chromatograms of caudate nucleus of the pig and caudate and lentiform nuclei of the cat in this figure revealed the presence of relatively

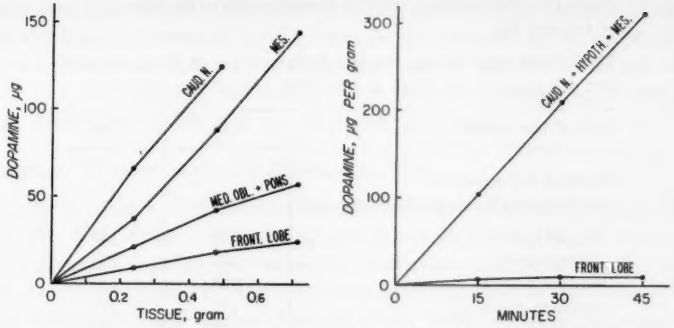

Figure 5. Apparent DOPA-Decarboxylase Activities of Extracts from Different Parts of the Cat's Brain.
To the left: Activity against enzyme concentration.
Incubation period: 45 minutes.
To the right: Activity against time of incubation.
Caud. N.: Caudate nucleus.
Mes.: Mesencephalon.
Front. lobe: Frontal lobe.
Hypoth.: Hypothalamus.
Med. obl.: Medulla oblongata.

large amounts of dopamine in these nuclei. As a further test of identity, the activation and flurorescence spectra of the oxidized eluates were always compared to those of synthetical noradrenaline and dopamine. Spectra obtained from the 2nd eluate from whole brain or from the caudate nucleus (Fig. 4) came very close to those of dopamine.

Determination of DOPA Decarboxylase Activity

The brain tissues were homogenized in 2.5 ml 1/15 M phosphate buffer, pH 7.5, per g tissue. The homogenates were centrifuged in a refrigerated centrifuge (0° C) at 15,000 × *g* for 15 min. An aliquot of the supernatant, generally 2.5 ml, and 0.1 mg pyridoxal phosphate in 0.1 ml water were placed into a Warburg cup. One mg l (—) DOPA in 0.25 ml of the phosphate buffer was put into one side arm of the cup. The cup was immersed in a water bath of 37° C. A stream of nitrogen was passed through the vessel under shaking for 10 min. The substrate was then tipped into the main vessel. The reaction was stopped after 45 min with 0.4 N $HClO_4$. The extract was made up to 10 ml with 0.4 N $HClO_4$ and centrifuged. The supernatant was neutralized to pH 6 with 2 N K_2CO_3 solution and the precipitate of potassium perchlorate spun down at 0° C. The extract was passed through a column containing Dowex 50 (0.15 g dry weight, mesh 200—400, X—4) which had been carefully buffered to pH 6 with M sodium acetate-acetic acid buffer. In order to remove the small amounts of DOPA retained by the resin at this pH, the column was washed with 10 ml 0.02 M phosphate buffer, pH 6.5. Elution was performed with 5 ml 2.4 N HCl. DOPA and dopamine were determined principally as described by Euler and Hamberg (1949) for determination of noradrenaline and adrenaline. As the concentration of dopamine in the eluate was too small to give satisfactory readings in this method it was somewhat modified. The eluate was partially neutralized with K_2CO_3 in substance and finally titrated to pH 6.5 with 2 N K_2CO_3. Two ml of the neutralized extract and 1 ml of

Table IV. Distribution of DOPA Decarboxylase in the Brain of Cat and Pig

The figures stand for μg dopamine formed per g tissue per hour and are mean values from at least four cats or two pigs

Part of the brain	Cat	Pig
Cerebral hemispheres (not corpus striatum and hippocampus)		
rostral part	48	140
caudal part	27	123
Caudate nucleus	288	342
Lentiform nucleus	101	269
Hippocampus	47	119
Hypothalamus	216	298
Diencephalon (not hypothalamus)	53	216
Mesencephalon	260	321
Pons	104	147
Medulla oblongata	115	148
Cerebellum	15	116

0.1 M phosphate buffer pH 6.5 were transferred to a test tube, and 0.2 ml 0.1 N iodine solution was added. After 4 minutes 1 ml sodium acetate-acetic acid buffer, pH 3.5, and 0.2 ml 0.1 N sodium thiosulphate solution were added. The extinction of the red product formed was immediately determined in a Beckman spectrophotometer at 530 mμ. The identity of the product with dopamine was confirmed by paper chromatography.

The accuracy of the method for estimation of dopamine in presence of large amounts of DOPA was tested by model experiments and recovery experiments in which known amounts of dopamine and DOPA were added to the extracts. The recoveries of dopamine were about 90 per cent. In some experiments the values obtained by determination of the carbon dioxide evolved and the amount of dopamine found in the extracts were compared. Only the brain parts with the highest enzyme activities could be used for this purpose. Such experiments using rabbit kidney cortex extracts were also performed. This tissue is rich in DOPA decarboxylase and the enzyme activity could be measured with good accuracy by means of the carbon dioxide evolved. The results obtained by the two methods agreed within a few per cent. In some experiments the influence of enzyme concentration and of incubation time was studied. As will be seen in Fig. 5 the amount of dopamine recovered is proportional to the concentration of enzyme and to the time of incubation.

The distribution of DOPA decarboxylase activity in various parts of the brains of pigs and cats was determined. The results are given in Table IV.

Results

Catechol Amines in Whole Brains of Various Species

Noradrenaline and dopamine were present in the brains of all the mammalian species examined (Table II). The dopamine content in the different species ranged from 0.19 μg (dog) to 0.60 μg (rat) per gram fresh tissue and was

generally somewhat higher than that of noradrenaline. The amount of adrenaline in the brains of several species examined appeared to be small. It did not seem to constitute more than a few per cent of the total catechol amine content. The accurate estimation of this amine in brain required a somewhat modified technique which will be described elsewhere.

Distribution of Catechol Amines in Mammalian Brain

The concentrations of noradrenaline and dopamine in various parts of the brain of the dog, sheep, pig and cat, are shown in Table III. It is apparent that noradrenaline was present in all the different parts of the brain, but at different concentrations. The highest values were recorded in the diencephalon and the midbrain. Still higher concentrations were obtained in some subdivisions of these parts. Low values were found in the cerebral cortex and the cerebellum. The distribution of dopamine differed greatly from that of noradrenaline, dopamine being found almost exclusively, three fourths, in the basal ganglia of the hemispheres. Small but possibly significant amounts of dopamine were recorded in other parts of the brain.

Distribution of DOPA Decarboxylase in Brain

As seen in Table IV the caudate and lentiform nuclei, which contained the highest dopamine content, also had high DOPA decarboxylase activities. High enzyme activity was also found in the brain stem where, with the exception of the mesencephalon, the levels of enzyme and noradrenaline concentration paralleled each other. The mesencephalon contained proportionally more of the enzyme compared to its catechol amine content than the rest of the brain stem. In the parts of the brain poor in catechol amines, namely the cerebellum and the cerebral hemispheres from which the caudate and lentiform nuclei had been removed, however, very little DOPA decarboxylase activity was recorded.

Discussion

The concentrations of noradrenaline in the different parts of the brain, as observed in the present paper are in fairly good agreement with those found by VOGT (1954). The fairly high amounts of adrenaline in the brains of mammals reported by VOGT (1954) and by MONTAGU (1957) could not be confirmed. In a few preliminary experiments appreciable amounts of this amine, however, have been recorded in the brains of amphibia (*Bufo vulgaris* and *Rana temporaria*). Also in other tissues of these animals the adrenaline forms a large porportion of the catechol amines (BERTLER and ROSENGREN 1959).

It is interesting to note the different distribution patterns of nordrenaline and dopamine in brain. The parts with the highest dopamine concentrations are the caudate and lentiform nuclei, where only small amounts of noradren-

aline can be detected. On the other hand the sites rich in noradrenaline (the hypothalamus and some other parts of the brain stem) contain but little dopamine. The distribution of dopamine differs also from that of noradrenaline in another way. Noradrenaline although concentrated in some regions is found all over the brain, whereas dopamine is confined to a few nuclei only. From the experiments on the distribution of DOPA decarboxylase it may be concluded that the catechol amines can be formed in the sites where they are found. The different distribution of noradrenaline and dopamine in the brain, as well as in peripheral organs of ruminants (BERTLER *et al.* 1959), suggests that dopamine not only is an intermediate in noradrenaline formation, but also has a function of its own.

The noradrenaline found in the brain may be present not only in the vasomotor fibres of the brain vessels, as first suggested by EULER (1946) and HOLTZ (1950), but also in the central representation of the sympathetic nervous system, as proposed by VOGT (1954).

From its localization to the corpus striatum it may be suggested that dopamine plays a part in the function of these nuclei which constitute an important unit of the extrapyramidal system. It is of interest to note that reserpine, a drug known to release both noradrenaline and dopamine from the cat and rabbit brains (HOLZBAUER and VOGT 1956, CARLSSON *et al.* 1957, CARLSSON *et al.* 1958), has a marked influence on motor acitivity and may cause a motor disturbance similar to Parkinson's syndrome. On the other hand excess of dopamine, induced by administration of DOPA to animals, causes motor hyperactivity (CARLSSON *et al.* 1958). There is thus evidence to suggest that the brain dopamine is involved in the control of motor functions. In view of the close functional relationship which appears to exist between the corpus striatum and the cerebral cortex, it does not seem impossible that the brain dopamine may influence other cortical functions as well.

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Acta physiol. scand. 1959. 47. 362—364

From the Department of Pharmacology, University of Lund, Lund, Sweden

Brain Catechol Amine Content after Sectioning the Adrenergic Nerves to the Brain Vessels

By

ÅKE BERTLER AND EVALD ROSENGREN

Received 25 May 1959

Abstract

BERTLER, Å. and E. ROSENGREN. Brain catechol amine content after sectioning the adrenergic nerves to the brain vessels. *Acta physiol. scand.* 1959. *47*. 362—364. — The catechol amine content of rat brains after extirpation of the superior cervical ganglia has been investigated, and the values compared to those obtained from a sham-operated group of animals. There was no difference between the two groups, suggesting that sympatho-adrenergic nerves do not significantly contribute to the brain catechol amine content in rats.

When noradrenaline and adrenaline were first demonstrated to occur in brain by EULER (1946), they were suggested to be present in the sympathetic nerve fibres of the brain vessels. This opinion was supported by HOLTZ (1950). VOGT (1954) reported, that noradrenaline and adrenaline were concentrated in certain autonomic centers of the brain stem, and suggested that the brain catechol amines were not exclusively localized in sympathetic nerves. This was supported by the observation in a few cats that the amount of noradrenaline in the hypothalamus was not affected by extirpation of the superior cervical ganglion. The possibility still remains open, however, that adrenergic nerves

Table 1. Noradrenaline (NA) and Dopamine (DA) in Rat Brain after Sympathetic Denervation

The amine values are given as μg per g tissue.

	No. of animals	Cerebral hemispheres		Brain stem	
		NA	DA	NA	DA
Control	6	0.29	2.15	0.60	0.17
	5	0.20	1.35	0.58	0.20
Average		0.25	1.75	0.59	0.19
Sympathectomized	6	0.24	1.47	0.72	0.44
	5	0.23	1.62	0.49	0.22
Average		0.24	1.55	0.61	0.33

contribute significantly to the noradrenaline content, particularly in such areas of the brain where the noradrenaline levels are relatively low, *e. g.* in the hemispheres. This possibility is investigated in the experiments described below.

Experimental

The experimental animals were 22 male and female rats weighing 140—195 g. The animals were divided into two equal groups. In one group the superior cervical sympathetic ganglion on both sides, and about one cm of the sympathetic chain adjacent to this ganglion were removed under ether anaesthesia. The rats of the other group were sham operated. Twelve days after operation the animals were killed by a blow on the neck and decapitated. Each brain was divided into the brain stem, the cerebral hemispheres and the cerebellum. The various brain parts of 5 to 6 animals in each group were pooled and examined together for their noradrenaline and dopamine contents according to methods described previously (Bertler, Carlsson and Rosengren 1958, Carlsson and Waldeck 1958).

Results and discussion

The concentrations of noradrenaline both in the cerebral hemispheres and in the brain stem were about the same in the denervated and the sham operated group (Table I). Nor was the amount of dopamine, which is present mainly in the cerebral hemispheres, affected by denervation. The concentration of catechol amines in the cerebellum was too small to be accurately estimated in these experiments. It may be concluded that the sympathetic nerves from the superior cervical ganglion do not contribute significantly to the noradrenaline content of the rat's brain. It therefore appears that the sympatho-adrenergic nerve supply of this organ is poor, if present at all.

This work has been supported by grants from the Medical Faculty of the University o Lund and by the Air Force Office of Scientific Research of the Air Research and Develf opment Command, United States Air Force.

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From the Department of Pharmacology, University of Uppsala, Sweden

Absorption of Solid Spherical Particles through the Intestinal Mucosa

By

LENNART JUHLIN

Received 25 May 1959

Abstract

JUHLIN, L. Absorption of solid spherical particle through the intestinal mucosa. *Acta physiol. scand.* 1959. *47*. 365—369. — Suspensions of spherical plastic particles in 5 per cent glucose solutions were given for drinking to mice and rats over a period of a week. The particles were prepared by emulsion polymerization of methyl methacrylate and marked with a fluoroscent colour. Batches of negatively and positively charged particles as well as hydrophilic and lipophilic particles of different sizes (0.01 μ—1.2 μ) were used. The particles should if they are absorbed in the intestines be carried to the blood stream and taken up by the reticulo-endothelial cells in the liver and spleen. However, no particles could be found in these organs by fluorescence determinations. If more than 2 parts per million of the ingested dose of negatively charged 0.07 μ particles had been absorbed they should have been detected. The difference between microparticles and microdrops, which can be deformed is stressed and its importance in the discussion of the mechanism of fat absorption has been pointed out.

One theory proposed to explain the mechanism of fat absorption is known as the Particulate Theory or Partition Hypothesis. It has been largely developed by FRAZER and his school (for ref. see FRAZER 1958, a, b). According to this concept the fat is emulsified in the intestinal lumen and absorbed as fat droplets. FRAZER found that liquid paraffin could be absorbed if pre-emulsified to a particle size of less than 0.5 μ. Negative results by LUNDBÆCK and MAALØE (1947) have been attributed to the instability of the emulsions employed (FRAZER 1958). PALAY and KARLIN (1956) interpret the morphological evidence

Table I. Size-distribution of particles. Preparation 2. 449 particles measured

Diameter μ	%	Diameter μ	%
<0.02	1.3	0.06—0.07	18.0
0.02—0.03	4.5	0.07—0.08	30.1
0.03—0.04	5.1	0.08—0.09	16.0
0.04—0.06	16.5	0.09—0.11	8.5

of fat absorption as an indication that particulate fat enters the columnar absorptive cells by a process akin to pinocytosis and is disgorged into the intercellular spaces by a reversal of the same mechanism. CLARK (1959) showed electron-microscopically, that proteins and various other colloidal materials administered orally to suckling rats and mice, were ingested by columnar absorptive cells of the jejunum and ileum. Approximately 18 days after birth the columnar cells lost the ability to ingest proteins and colloids and no longer contained vacuoles and numerous tubules. However, CLARK (1959) agreed with PALAY and KARLIN (1956) "that pinocytosis, at least on a small scale, may occur in the columnar absorptive cells throughout life". "Adult animals may not have lost the capacity for pinocytosis but rather have become selective as to what substances provoke it." The divergent ideas on fat absorption were discussed at a conference in 1955 by, among others, Frazer, Bergström and Borgström (POPJÁK and LE BRETON, 1956).

Fat globules are fluid and in the presence of surface active agents the forces that are needed to deform the spherical shape may theoretically be infinitely small. They could therefore be thought to flow through extremely small pores. Therefore it seemed to be of interest to investigate whether solid particles could be absorbed. Absorbed particles which have reached the blood should be removed by the reticulo-endothelial system especially in the liver and spleen, where they are retained for long periods and are easily estimated fluorimetrically (JUHLIN 1958, 1959).

In the present experiments positively and negatively charged particles with sizes between 0.02 μ and 1 μ and with hydrophilic or lipophilic properties were used. None of them was absorbed.

Methods

Particles. The particles were prepared by emulsion polymerisation of methyl methacrylate and marked with a fluorescent dye as described earlier (JUHLIN 1956, 1959). The following particles were used:

1. Negatively charged hydrophilic particles with a mean diameter of 0.1 μ (Preparation Sh, JUHLIN 1956).
2. Negatively charged hydrophilic particles with a mean diameter of 0.07 μ. Prepared as Preparation Sh, but the amount of sodiumlauryl sulphate was increased from 3 g to 9 g. The size distribution is given in Table I.

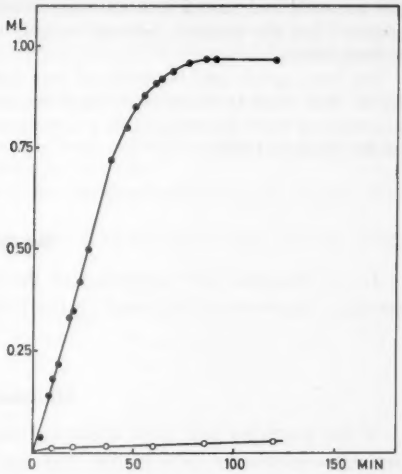

Fig. 1. Estimation of water absorption of particle-powder according to ENSLIN.

Abscissa: Time in minutes

Ordinate: Amount of water absorbed in ml.

•———• Hydrophilic particles. Preparation Lh.

o———o Lipophilic particles. Preparation L1.

3. Negatively charged hydrophilic particles with a mean diameter of 0.8 μ (Preparation Lh, JUHLIN 1956).

4. Positively charged hydrophilic particles with a mean diameter of 0.05 μ (Preparation see JUHLIN 1959).

5. Negatively charged lipophilic particles with a mean diameter of 0.8 μ (range 0.2—1.2 μ). (Preparation L1, JUHLIN 1956.) These particles had a Hydrophilic-Lipophilic balance (HLB) value of 4.7 according to GRIFFIN (1949 and 1954). To get an impression of what such a HLB value practically means the water uptake of the particle-powder was measured with an Enslin appratus (ENSLIN 1936). In this apparatus the powder is brought into contact with water and the rate of water absorption of the powder is measured. For comparison hydrophilic particles (HLB 9.8) of the same size (Preparation Lh) were also tested. The difference in water uptake into the air spaces between the particles is shown in Fig. 1 and demonstrates the difference in wettability.

Experimental procedure. Male albino rats (200 g) and mice (20 g) were placed singly in cages. In one group of rats 100 g of each of the 5 types of particles in a suspension of 12 per cent by weight of solids with glucose added to 5 per cent was given in place of drinking water. The dose was consumed within 4 to 5 days. Two rats were used for each type of particle. Another similar series of 10 rats were given, in addition to the glucose solution with particles rat bread *ad lib.*[1] They consumed their particles within 5 to 7 days.

In a third series mice were given 50 g of each type of particles as in the first rat group. Two mice were used for each type of particles. The particle suspension had been consumed within 6 to 8 days.

When the animals had ingested their particle suspension they were killed and the internal organs dissected free and examined under ultraviolet light. The particle fluorescence was visible only in the stomach and the intestines. When the intestinal content

[1] The bread contained 32 % wheat-meal, 25 % corn meal, 6 % linseed meal, 3 % Lucerne powder, 15 % meat powder, 3 % dry brewer's yeast, 2.6 % wheat-germ, 0.4 % iodized table salt, 1 % ground lime stone and 10 ml stabilized A and D vitamin concentrate per 100 kg bread.

was removed and placed in a test-tube it was seen that some of the particles had aggregated but the majority behaved roughly as non-ingested particles with respect to sedimentation.

The liver, spleen and in almost all rats those lymph-nodes from the thoraco-lumbar region that could be found were taken out and their particle content was estimated by determining their fluorescence in a Coleman Electronic Photofluorometer as described earlier (JUHLIN 1958).

Results

In no instance after ingestion of the different types of particles could any particle fluorescence be found in the liver, spleen or lymph-nodes.

Discussion

If the particles had been absorbed they should have been taken up by the reticulo-endothelial cells in the liver and spleen and to the greatest part have remained there during the experimental period (JUHLIN 1960). With the method used as little as 50 μg/g tissue of the negatively charged small particles can be estimated with accuracy. Due to a lower fluorescence content in the other types of particles an absorption of about 500 μg/g tissue should have been measurable. This means that if as much as 0.002—0.001 % of the given dose had been absorbed it should have been detected. Positive results would have supported the particulate theory of FRAZER. The results obtained can not, however, be used as an argument against this theory since there is a difference between the solid microparticles and the deformable microdrops.

Thanks are due to Mrs. ASTA PALIS for valuable technical assistance and to Dr. B. AF EKENSTAM and Mr. A. FURENDAL, A.-B. Nobelkrut, Bofors, Sweden, for preparation of the particles, and for measuring their wettability.

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Acta physiol. scand. 1959. 47. 370—374

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Intrinsic Factor and Radiovitamin B_{12} Excretion in Rats

By

RALPH GRÄSBECK, LARS RUNEBERG and KAI SIMONS

Received 27 May 1959

Abstract

GRÄSBECK, R., L. RUNEBERG and K. SIMONS, Intrinsic factor and radiovitamin B_{12} excretion in rats. *Acta physiol. scand.* 1959. *47*. 370—374. — The urinary and fecal excretion of radioactive material was studied in normal and gastrectomized rats after parenteral injection of radiovitamin B_{12} alone or in conjunction with rat or hog intrinsic factor preparation. The gastrectomized animals excreted more radioactivity with the feces (but not with the urine) than the normal animals when the vitamin was injected either alone or with rat intrinsic factor. This indicates that the reabsorption of vitamin B_{12} excreted into the intestine (*e. g.* with the bile) needs the presence of the gastric intrinsic factor, and that at least a part of the excreted vitamin remains in the form of vitamin B_{12}. In all groups the excretion pattern was the same irrespective of whether the vitamin was injected alone or with rat or hog intrinsic factor. This shows that the tissues are able rapidly to liberate vitamin B_{12} bound to intrinsic factor preparations. No support is obtained for the view that intrinsic factor is absorbed from the intestine.

This study was undertaken for two reasons. Firstly, intrinsic factor preparations have been found to stimulate the uptake of vitamin B_{12} in liver slices in vitro (MILLER and HUNTER 1957). Should such preparations exert the same effect in vivo, the excretion pattern of radioactive vitamin B_{12} would be expected to be different according to whether the vitamin is injected parenterally with intrinsic factor or is administered alone. Secondly, previous experiments indicated that vitamin B_{12} is subject to an enterohepatic circulation,

biliary radioactivity being reabsorbed in the intestine after parenteral injection of radioactive B_{12} (GRÄSBECK, NYBERG and REIZENSTEIN 1958). If intrinsic factor is needed for this reabsorption, more radioactivity should be eliminated with the feces of gastrectomized rats than with those of normal rats in experiments similar to those described above.

Material and Methods

20 mμg of Co^{58} labeled vitamin B_{12} (obtained from the Radiochemical Centre, Amersham, England), corresponding to 46 mμc at the beginning of the experiments, were injected intraperitoneally in a single dose with or without added intrinsic factor preparation into normal or gastrectomized rats of the Wistar strain. The animals were kept in metabolic cages, and their excreta were collected three times daily for periods up to 14 days after the injection. The excreta were dried and ashed at 600° C, whereupon the ashes were packed tightly into test-tubes of equal size. The contents of the tubes were adjusted to the same volume by mixing inert powder with the ashes. The gastrectomized rats had been subjected to total gastrectomy by a technique described elsewhere (GRÄSBECK 1959). In order to adjust the body stores of vitamin B_{12} to approximately the same level, all rats received three subcutaneous injections of 1 μg nonradioactive B_{12} before the first experimental period, the last injection being given one week before the radiovitamin B_{12} injection. Because of the shortage of rats, the same animals had to be used once more. After the first experimental period, 1 μg B_{12} was injected subcutaneously and after two weeks the excreta were checked for residual radioactivity. As this was negligible, a new experimental period was started three weeks after the end of the first period.

Since our excretion figures are comparable to those found in previous studies on rats (OKUDA, GRÄSBECK and CHOW 1958), it is unlikely that use of a vitamin B_{12} deficient diet or omission of the parenteral injections of nonradioactive B_{12} would have greatly influenced the present results.

Two intrinsic factor preparations were administered: A lyophilized rat stomach mucosa preparation, prepared as described by GRÄSBECK (1959), and a purified hog stomach preparation BFS 209 (obtained through the courtesy of N. V. Organon, Holland). The latter preparation was active in the Schilling test in 5 mg dose, stimulated the uptake of radiovitamin B_{12} in rat liver homogenates in a concentration of 15 μg/ml, and bound 0.27 μg B_{12}/mg. All preparations were administered in physiological saline and injected in 2 ml volumes. The amount of intrinsic factor preparation administered was such that when the dose was mixed with the radiovitamin B_{12} prior to injection, all the vitamin was expected to be found.

In order to rule out liver damage as a cause of increased fecal excretion in the gastrectomized rats, after discontinuation of the experiments these animals were killed and their livers subjected to conventional histologic examination. No signs of liver damage were found.

Results

The experimental data on the urinary and fecal elimination of radioactive material in normal and gastrectomized rats are given in Fig. 1. The general excretion pattern seen in the present study was in agreement with that observed previously (OKUDA *et al.* 1958), the urinary excretion dominating in the begin-

Fig. 1. The elimination of radioactive material after parenteral injection of 20 mμg of radiovitamin B_{12} alone or in conjunction with intrinsic factor preparation (IF). The short horizontal lines indicate ranges. Material administered: Period 1. Group A (4 normal rats) and D (3 gastrectomized) received no IF, Group B (5 normal) 20 mg rat IF, and Group C (4 normal) 0.5 mg hog IF. Period 2. Group A no IF, Group B 0.1 mg hog IF, Groups C and E (gastrectomized) 25 mg rat IF.
Period 1, day 1, urinary excretion data unreliable owing to technical errors.

ning of the experimental periods, whereas later on the fecal excretion exceeded urinary elimination. The mean fecal excretion values were considerably increased in the gastrectomized group as compared with the normal animals both when the radiovitamin was administered alone and when it was given with rat intrinsic factor. The difference between the fecal excretions of the gastrectomized group and all other groups was statistically highly significant for all collection periods, except the first day after injection. The urinary excretion of the gastrectomized group did not differ significantly from that of the other groups.

Neither the fecal nor the urinary excretions differed significantly from each other in the three remaining groups, viz. the control group and the two groups receiving the radiovitamin bound to intrinsic factor preparations.

Discussion

The increased fecal elimination of radioactive material in the gastrectomized group strongly suggests that the gastric intrinsic factor is necessary for the intestinal reabsorption of vitamin B_{12} excreted into the intestinal contents (from sources such as the bile). The difference between the fecal excretions of the gastrectomized and normal groups gives an indication of the magnitude of the intestinal reabsorption of vitamin B_{12} in normal rats. Because intrinsic factor is needed for its reabsorption, at least this fraction of the radioactive material excreted into the intestine seems to have remained in the form of unaltered vitamin B_{12}.

It is somewhat surprising to note that the administration of intrinsic factor — homologous and heterologous — did not cause any significant increase in the retention of the parenterally administered radioactive vitamin. Had this been true only for the normal rats, the possibility would have remained that absorbed gastric intrinsic factor circulating in the blood, as suggested by RANEY, HANSEN and MILLER (1959), had made the controls behave in the same way as the animals receiving intrinsic factor. Lack of circulating intrinsic factor, necessary for the fixation of absorbed vitamin in the tissues, would have explained the increased fecal elimination of the vitamin in the gastrectomized group. However, this hypothesis is unlikely to be correct, because the urinary excretion was normal in the gastrectomized group, and the fecal excretion was not normalized by parenteral administration of rat intrinsic factor.

Thus this study does not support the view (RANEY *et al.* 1959) that intrinsic factor is absorbed from the intestine. On the whole, there is little evidence in favor of this suggestion. Most of the supporting evidence has been obtained with heterologous systems (rat liver + hog intrinsic factor, etc.). Since in man vitamin B_{12} administered parenterally is effective in genuine pernicious anemia and in megaloblastic anemia following total gastrectomy, circulating gastric intrinsic factor cannot be indispensable for the tissue utilization of the vitamin. There remains only one indication that intrinsic factor is absorbed, namely, that antibodies against intrinsic factor are present in the serum of pernicious anemia patients who have become refractory towards hog intrinsic factor (SCHWARTZ 1958).

Since the excretion pattern of radiovitamin B_{12} injected alone or in conjunction with intrinsic factor was the same from the very start of the experimental periods, rat tissues must contain enzyme systems capable of liberating vitamin B_{12} bound to rat or hog intrinsic factor preparations.

Conclusions

In the rat the gastric intrinsic factor is necessary for the reabsorption of vitamin B_{12} excreted into the intestinal contents. Part of the vitamin excreted into the intestine remains in the form of unchanged vitamin B_{12}. This study does not support the view that intrinsic factor is absorbed from the intestine. Rat tissues are able rapidly to liberate vitamin B_{12} bound to rat or hog intrinsic factor preparations.

We are greatly indebted to DR. PER FORTELIUS for making the histologic examination of the livers of the gastrectomized rats. This study was supported by a grant from Sigrid Jusélius Foundation.

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Acta physiol. scand. 1959. 47. 375—382

From the Institute of Occupational Health, Helsinki, Finland

Transfer of Thiamine across the Placenta of Guinea Pig

By

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Received 4 June 1959

Abstract

Brink, C., R. Esilä, M. J. Karvonen and A. Laamanen. Transfer of thiamine across the placenta of guinea pig. *Acta physiol. scand.* 1959. *47.* 375—382. — Thiamine is present in human foetal blood at a concentration higher than in maternal blood. The mechanism of keeping up such a gradient is not known. The purpose of the present study was to determine, whether an injection of thiamine into the mother would affect the thiamine level in foetal blood or plasma, and *vice versa.*

The pre-experimental level of thiamine in foetal blood did not differ systematically from that in maternal blood. When large amounts of thiamine were injected, the level rose slightly also on the other side of placenta, but this rise was very much less than on the side of the injection. This indicates a very small net transfer of thiamine across placenta. The permeability of placenta to thiamine differs basically from that to several other substances.

The concentration of several vitamins has been shown to be higher in foetal than in maternal blood. This applies to vitamin C (Elmby and Becker-Christensen 1938, Lund and Kimble 1943, Hamil *et al.* 1947), to vitamin B_2 (Hagerman and Villee 1954), and to vitamin B_1 (Gaehtgens 1941, Neuweiler 1940, 1941, Hildebrandt and Abderhalden 1942—1943 a, Slobody, Willner and Mestern 1949).

The presence of a substance in the blood of the foetus at a concentration higher than in that of the mother may result from the synthesis of the substance on the foetal side or from the "pumping" action of the placenta. How-

ever, there are also other possibilities. Thus, it has been demonstrated (LUST, HAGERMAN and VILLEE 1954) that although the total content of riboflavin is higher in the human foetal serum, the concentration of flavin adenine dinucleotide (FAD) is less in the foetal than in the maternal serum. Neither of these probably enters foetal blood through diffusion; FAD is present at quite high a concentration in placenta, and riboflavin is likely to be split from FAD by enzymatic processes.

A mechanism of another nature seems to be responsible for the higher blood concentration of vitamin C on the foetal side (RÄIHÄ 1959). The content of ascorbic acid (AA) is higher in foetal than maternal blood, but the gradient of dehydroascorbic acid (DHA) is in the opposite direction. It appears that DHA is transferred from the maternal to the foetal blood along a concentration gradient and is then converted to AA, for which placenta is much less permeable than for DHA.

Thiamine occurs in the blood in three forms, phosphorylated as cocarboxylase (CC), in the free form (FT), and as a phosphoric acid-protein-complex. FT is found in plasma, whereas in the cells thiamine is present as CC and as combined with protein (DIXON 1951, BURCH *et al.* 1952).

Experiments in which the thiamine level in maternal and foetal level are followed, while thiamine is administered to the mother, are likely to clarify the mechanism of the placental transfer of thiamine. A series of such experiments were made on guinea pigs.

Methods

The experiments were made on pregnant guinea pigs under nembutal anaesthesia, exposing the foetuses through a caesarean section in physiological saline at 37° C, while still connected with placenta. The general arrangement was the same as published by KARVONEN and RÄIHÄ (1954). Fifteen pregnant guinea pigs were used; the number of foetuses was most commonly 3.

The mothers were given a slow constant speed injection of 10 ml of a 0.01, 0.1, or 1.0 per cent solution of thiamine hydrochloride in 0.9 per cent sodium chloride. The injection lasted generally 8 min. When studying the transfer of thiamine from foetus to mother, 2.0 ml of a 1.0 per cent solution of thiamine was injected in a short time into the umbilical vein.

Blood samples were taken from the carotid artery of the mother and from the umbilical vein of the foetuses before, during, and after the infusion.

Free thiamine was determined with a micro modification of the thiochrome method (HAWK, OSER and SUMMERSON 1954, Methods of Vitamin Assay 1951). Samples of 0.2 ml blood were used; they were haemolysed with 2.3 ml distilled water. The haemolysate was acidified with 1.0 ml of 1.0 N HCl. Protein was precipitated with 5.0 ml of 10 per cent HPO_3, after which water was added ad 25 ml. The solution was centrifuged for 15 min, and 20 ml of the supernatant was allowed to flow slowly through adsorption tubes filled with Permutit T. The columns were washed twice with 10 ml of warm water and then eluted with 12.5 ml of acidified 25 per cent KCl solution (8.5 ml conc. HCl added to 1 litre of 25 per cent KCl).

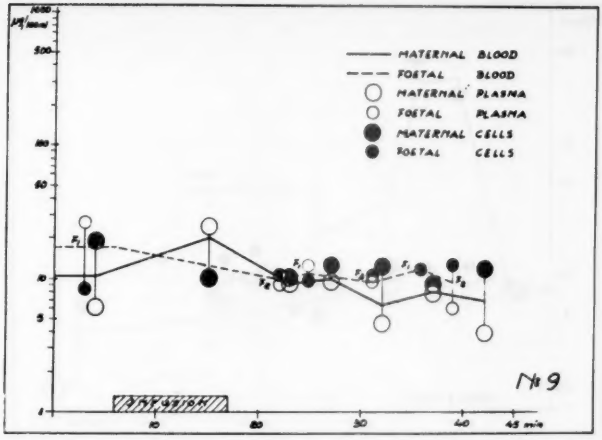

Fig. 1. Free thiamine in the blood, cells and plasma of maternal and foetal guinea pigs, on injecting 1.0 mg thiamine into the maternal circulation.

Thiamine in 2.5 ml of the eluate was oxidized to thiochrome by adding 1.5 ml of 10 per cent $K_3Fe(CN)_6$. The solution was shaken vigorously for 90 sec with 7.5 ml of isobutanol. The aqueous layer was discarded, and the isobutanol dried by adding anhydrous sodium sulphate. After filtration, the concentration of thiochrome was determined fluorometrically, with Beckman photoelectric colorimeter.

Separate determinations were made on blood and red cells. Because of the tendency of foetal blood to spontaneous hemolysis, determinations on plasma were not carried out. Plasma concentrations were calculated from the above values with the aid of the hematocrit readings.

Results

Normal concentrations of thiamine. The concentration of FT in the blood and plasma of maternal and foetal guinea pigs, before the administration of thiamine, is shown in Table I. There was no systematical gradient between maternal and foetal blood. In 8 experiments, the foetal blood showed a higher concentration of FT, in one experiment the values were the same, and in 6 experiments the maternal level was the higher one. In five of the six experiments in which separate values for plasma were obtained, the foetal level was higher than the maternal one.

Maternal injections of thiamine. An injection of 1.0 mg thiamine (10 ml of 0.01 per cent solution, exp. no. 9) led to a considerable increase of maternal blood and plasma FT levels, but to no increase in foetal blood or plasma (Fig. 1). The content of FT in the maternal cells showed a gradual increase, from 10.8 during infusion to 13.1 μg per 100 ml, while the rise in the maternal plasma rapidly passed.

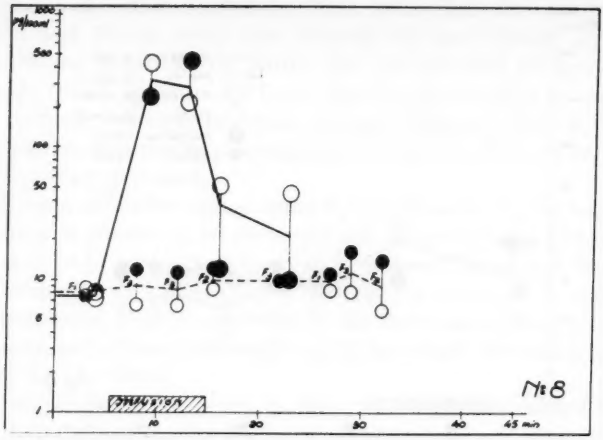

Fig. 2. Free thiamine in the blood, cells and plasma of maternal and foetal guinea pigs, on injecting 10 mg thiamine into the maternal circulation.

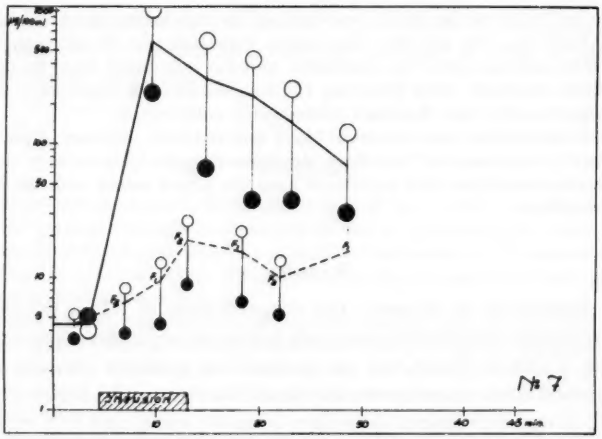

Fig. 3. Free thiamine in the blood, cells and plasma of maternal and foetal guinea pigs, on injecting 100 mg thiamine into the maternal circulation.

An injection of 10 mg thiamine (10 ml of 0.1 per cent solution, exp. no. 8, 10 and 15) increased the level of FT in maternal blood to more than 300 μg per 100 ml (Fig. 2). In foetal blood, FT rose much less, at a maximum by 4.5 μg per 100 ml. The content of thiamine again rose first in maternal plasma, later in cells. After injection, the maternal values in whole blood, plasma and

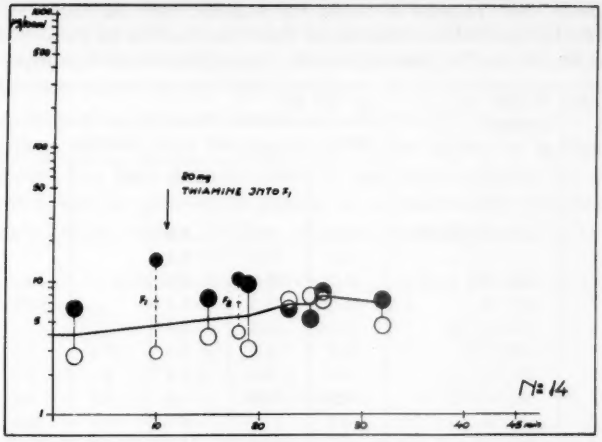

Fig. 4. Free thiamine in the blood, cells and plasma of maternal and foetal guinea pigs, on injecting 20 mg thiamine into the foetal circulation.

cells rapidly decreased. The concentration in plasma remained at this stage higher than in the cells.

An injection of 100 mg thiamine (10 ml of 1.0 per cent solution, exp. no. 6 and 7) resulted in maternal blood levels of the order of 600—700 μg per 100 ml, and corresponding plasma levels up to more than 1,000 μg per 100 ml (Fig. 3). The foetal levels also increased, but very much less, the maximum plasma concentration remaining below 70 μg per 100 ml.

Foetal injections of thiamine. An injection of 20 mg thiamine into a foetus (2.0 ml of 1.0 per cent solution, exp. no. 12 and 14) resulted in a slow, distinct rise of the level of free thiamine in maternal whole blood and plasma (Fig. 4). At the same time, the level of thiamine in the blood of the injected foetus was, of course, very high; a value of 6,000 μg per 100 ml was recorded.

Discussion

The normal level of thiamine in human blood is from 3 to 9 μg per 100 ml (WRIGHT 1952, p. 1030). In nine of the fifteen experiments, the maternal blood level exceeded this range before the administration of thiamine. Evidently the guinea pigs' ration contained adequate amounts of thiamine.

The level of thiamine in the foetal *blood* was of the same order as in maternal blood (Table I). According to several investigators, the thiamine level in human foetal blood is distinctly higher than in maternal blood. In this respect the findings in man and guinea pig differ from each other.

The foetal *plasma* levels, however, were in 5 of the 6 cases examined higher

Table I. Level of Free Thiamine in Guinea Pig Maternal (M) and Foetal (F) Blood and Plasma, and the Difference between Maternal and Foetal Blood and Plasma Values (M—F) Expressed as μg per 100 ml. The Values Refer to the Foetus Whose Weight is in Italics

No.	Number of foetuses	Weight of foetuses g	μg/100 ml					
			Blood			Plasma		
			M	F	M—F	M	F	M—F
1	4	*106*, 69, 84, 96	10.0	11.3	− 1.3	..	..	..
2	3	.., .., ..	12.9	17.1	− 4.2	..	..	..
3	2	*92*, 108	21.3	19.6	+ 1.7	..	..	..
4	3	*46*, 58	12.0	13.4	− 1.4	..	..	..
5	3	*34*, 62, 55	7.5	13.3	− 5.8	..	..	..
6	2	*130*, 135	11.9	6.6	+ 5.3	13.4	4.5	+ 8.9
7	2	*90*, 95	4.6	4.6	± 0.0	4.1	5.3	− 1.2
8	3	*58*, 57, 62	7.9	8.3	− 0.4	8.1	8.6	− 0.5
9	3	*66*, 55, 56	10.8	17.7	− 6.9	6.1	26.9	− 20.8
10	1	*85*	11.5	12.3	− 0.8	13.2	14.4	− 1.2
11	3	*100*, 95, 90	17.1	12.9	+ 4.2	..	8.4	..
12	3	*50*, 50, 55	6.5	4.6	+ 1.9	1.7	3.1	− 1.4
13	2	.., ..	5.3	4.4	+ 0.9	..	..	..
14	2	*40*, 40	4.1	7.1	− 3.0	..	..	..
15	2	*65*, 65	9.9	7.4	+ 2.5	..	..	..
Average			10.2	11.4	− 0.5	7.8	10.2	− 2.7

than the corresponding maternal plasma values. Evidently the placenta of the guinea pig is able to keep the level of thiamine higher in the foetal than in the maternal plasma.

When either the maternal or the foetal side of the placenta were flooded with unphysiological concentrations of thiamine, its level rose also on the other side of placenta. However, the levels on the two sides of placenta remained very far from equal. It thus appears that placenta is rather poorly permeable to FT. The rises observed suggest a slight penetration of thiamine; this may be verified with the aid of labelled thiamine.

At delivery, the concentration of thiamine has been found to be higher in human umbilical venous than in umbilical arterial blood (HILDEBRANDT 1940, GAEHTGENS). If such a considerable difference would represent the situation throughout pregnancy, very large amounts of thiamine would be transferred all the time from the mother to the foetus. A constant flow of thiamine could be due to a rapid metabolism of it in the foetus. This, however, appears unlikely. Since there also is no marked storage of thiamine in the foetal organism (HILDEBRANDT and ABDERHALDEN 1942—1943 b) it is suggested that the observed umbilical arterio-venous differences have been connected with the delivery and are not typical of foetal life. Further work is evidently needed for establishing this claim.

The penetration of placenta by thiamine is of quite another type than that by monosaccharides such as glucose or d-xylose. These sugars pass across placenta rapidly, but never against a concentration gradient, whereas thiamine crosses placenta rather slowly, but appears to do it also against a gradient. In both cases, "active" processes evidently are involved. For thiamine, a "pumping" action of the placenta must be assumed; for the sugars, on the other hand, no "pumping" has been demonstrated, but an active selection by placenta is demonstrated by its poor permeability to several other monosaccharides, including the optical isomer l-xylose (KARVONEN and RÄIHÄ 1957).

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On the Mechanisms of Dark Adaptation in Day- and Night-Insects

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The present investigations were carried out in order to obtain information concerning the mechanisms of dark adaptation in day- and night-insects. To the authors' knowledge there are no earlier reports on comparative functional studies on insects representative for the two groups. Diurnal butterflies (Erebia, Argynnis, Vanessa) and nocturnal moths (Cerapteryx, Rhyacia, Geometra, Hepiolus) belonging to Lepidoptera were chosen. Threshold determinations of the monophasic electrical receptor response to 1/5 sec test flashes were made during 30—40 min after the cessation of pre-adapting illumination (3 minutes with a light intensity giving maximal response). The curve for dark adaptation in the diurnal Lepidoptera tested was found to be perfectly smooth and the range of the adaptive change usually did not exceed 1 log unit. The dark adaptation of the nocturnal moths tested, on the other hand, was found to proceed in two steps. The first step covered a range of change in threshold of 1—1.5 log units and was followed by a prolonged second phase covering 1—3 log units the latency of which, measured from the beginning of the dark adaptation, was different in different species (from 3—4 min in Geometra to 18 min in Hepiolus). The discontinuous curve which was found in the nocturnal moths was of the same type as that of the extrafoveal vertebrate adaptation curve, the two consecutive phases of which are ascribed to changes in sensitivity of cones and rods respectively. Since both structural (Cajal and Sanchez 1915, Hanström 1927) and electrophysiological (Autrum and Stumpf 1953, Walther and Dodt 1957) studies indicate the existence of different types of visual cells in the compound eye it would be tempting to ascribe the two phases to threshold changes in receptors of different functional characteristics. However, it is also well known that gross movements of retinal

pigments are characteristic of many nocturnal insects (see PARKER 1932). Thus, in the light-adapted state the ommatidia are ensheathed by pigment whereas in the dark-adapted state the pigment is retracted allowing incident rays from several facets to reach one ommatidium. A close relation between the pigmentary migration and the increase in sensitivity may therefore be assumed. In order to test this assumption comparative studies were made of the pigment migration and the change in sensitivity during dark adaptation in the nocturnal moth Cerapteryx graminis. Light-adapted eyes as well as eyes which had been dark-adapted during varying periods ranging from 1 to 30 min were fixed in Bouin-Duboscq-Brasil's mixture and stained with hemalum-eosin (see TUURALA 1954). Using this procedure the pigment migration could easily be followed during the course of dark adaptation. The material so far obtained showed no significant pigment migration during the first phase of the dark adaptation curve. The pigment retraction started at about the same time as the second phase of the dark adaptation curve and proceeded in parallel with the increase of sensitivity during the second phase. In preparations from nocturnal species with a normal first phase in which the second phase of the dark adaptation curve was reduced (probably due to injury) the pigment migration was found to be impaired.

The investigations show that the huge increase of sensitivity signalled by the second phase of the dark adaptation curve in nocturnal insects is connected with the pigment migration which underlies the transition of the compound eye from the apposition into the superposition state.

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